Placental Growth Factor (PlGF) Regulation and Expression in Endothelial Cells

By

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This PhD thesis written in 2016 is dedicated:

To My beloved parents, Dr S K Lal and Flora Grace

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Abstract

The role of Placenta growth factor (PlGF) in physiological angiogenesis is quiescent, preclinical and human studies have established PlGF angiogenic activity to be regulated in pathologies. PlGF elicits its cellular functions via (vascular endothelial growth factor receptor) VEGFR-1 alone, and has gained more attention for its safety profile, especially when compared to the risks involved in VEGF-derived therapies. Loss and gain studies of PlGF function highlight the contribution of PlGF in disease aggravation or alleviation; however, factors regulating PlGF expression remains elusive.

Data from this study demonstrates basic fibroblast growth factor (FGF-2) induced expression of PlGF in cultured ECs. FGF-2-amplified PlGF via transcriptional regulation, demonstrated a significant increase in both PlGF protein and mRNA levels. Using pharmacological inhibitors and adenoviral constructs to modulate signalling enzymes, I established that the FGF-2-amplified EC expression of PlGF involved multiple signalling cascades; namely the Ras/Raf/MAPK, protein kinase C (PKC), and phospholipase D (PLD) signal transduction pathways. Functional studies conducted with PlGF gene knock-down in endothelial cells (EC) displayed reduced FGF-2-mediated angiogenic activity in *invitro* tube formation. This finding was consolidated using the mouse aortic ring assay; significant reduction in sprouting angiogenesis was noted in FGF-2 treated aortic tissue from null mice ($plgf^{-/-}$) compared to their wild-type controls.

Besides being a pro-angiogenic factor, PlGF is also a strong pro-inflammatory mediator. Chronic inflammation is a hallmark of several inflammatory disorders and plays a key role in the pathogenesis of endothelial dysfunction. In this study, decline in endothelial PlGF protein levels in ECs in response to pro-inflammatory cytokines TNF-α, IL-1β, and IFNγ has been revealed. Real-time PCR and PlGF promoter activity studies confirmed TNF-α reduced endothelial PlGF expression to be transcriptionally regulated. Endothelial dysfunction has been reported to be mediated by EC apoptosis that may eventually lead to systemic vascular dysfunction (SVD), a common denominator for several inflammatory and chronic disorders. Loss of PlGF significantly increased EC apoptosis even in the presence of high serum concentrations. Additionally, exogenous growth factors could not rescue EC apoptosis in PlGF gene knock-down HUVEC. Data from these studies identified a new pathway leading to EC apoptosis, secondary to PlGF loss or inhibition in response to the "alarming cytokines" TNF-α, IL-1β.

Collectively, these data demonstrate the factors that regulate endothelial PlGF expression and contribute a vital discovery of new biological functions of endothelial PlGF in FGF-2-mediated angiogenesis. These findings add new dimensions to the regulatory mechanisms governing endothelial PlGF that may have significant implications for therapeutic approaches aimed at promoting or disrupting neovascularisation.

Keywords: PlGF, FGF-2, neovascularisation, VEGFR-1, TNF-α, functional assay, endothelial apoptosis, endothelial dysfunction

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Ad	Non-replicative, recombinant adenovirus.
ApoE$^{-/-}$	Apolipoprotein-E-deficient
aPKC	Abnormal protein kinase C
BAEC	Bovine aortic endothelial cells
Bp	Base pairs
FGF-2	Basic fibroblasts growth factor
BSA	Bovine serum albumin
BWH	Birmingham women's hospital
CVD	Cardiovascular disease
DAG	Diacylglycerol
DBD DNA	Binding-Domain
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulphoxide
Dn-	Dominant-negative
DTT	Dithiothreitol
EC	Endothelial cells
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix

EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-Linked Immunosorbent Assay
eNOS	Endothelial nitric oxide synthase
EPC	Endothelial progenitor cells
ETS	Domain transcription factors
ERK	Extracellular signal regulated kinase a.k.a MAPK
ET-1	Endothelin-1
FAT	Factor acetyl transferase
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
g	Gravity
G6Pase	Glucose-6-phosphatase
GPCRs	G protein-coupled receptors
G/P/S	L-Glutamine, 10U/ml penicillin and 0.1 μg/ml streptomycin
GLUT-4	Glucose transporter type 4
HAEC	Human aortic endothelial cells
HBSS	Hank's balanced saline solution
HEK 293	Human embryonic kidney 293 cells

ICAM-1	Inter-Cellular Adhesion Molecule 1
IGFPB	Iinsulin-like-growth factor binding proteins
HIF-1	Hypoxia Inducible Factor-1
H_2O_2	Hydrogen peroxide
HRP	Horseradish peroxidase
HUVEC	Human umbilical vein endothelial cells
HG	Hyperglycaemia
ifu	Infectious unit
IG	Immunoglobulin
IGF-1	Insulin-like growth factor-1
IGF-IR	Insulin-like growth factor-1 receptor
IP	Immunoprecipitation
IR	Insulin receptor
IRS	Insulin/IGF-I receptor substrates
IL-2	interleukin-2
IL-6	interleukin-6
JNK	c-Jun N-terminal kinases
kDa	Kilo Dalton
LPL	Lipoprotein lipase

M199	Medium 199
MEK	Map kinase kinase
MOI	Multiplicity of infection
Myr	Myristoylated
NES	Nuclear export sequence
NLS	Nuclear localisation signal
NF-KB	Nuclear Factor-k B
NO	Nitric oxide
OD	Optical density
OS	Osmolarity control
NP-1	Neuropilin-1
NP-2	Neuropilin-2
PAI-I	Plasminogen activator inhibitor-I
PBS	Phosphate Buffered Saline
PDGF	Platelet-derived growth factor
PDK1	Phosphoinositide-dependent protein kinase 1
PI3K	Phosphoinositide 3-kinase
PI(3)P	Phosphatidylinositol 3-phosphate
PIP2	Phosphatidylinositol (3,4)-bisphosphate ($PI(3,4)P_2$)

PIP3	Phosphatidylinositol (3,4,5)-trisphosphate ($PI(3,4,5)P_3$
PlGF	Placental growth factor
PKB	Protein kinase B
PKC	Protein kinase C
PLCγ	Phospholipase Cγ
PTEN	Phosphatase and tensin homolog on chromosome 10
PTPases	Phosphatases
qPCR	Quantitative polymerase chain reaction
RA	Rheumatoid arthritis
MAPK	Mitogen-activated protein kinase
RIPA	Radio immunoprecipitation assay
ROS	Reactive oxygen species
RPE	Retinal pigment epithelium cells
RT	Room temperature
RTKs	Receptor tyrosine kinases
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
siRNA	Short interfeing RNA
SOS	Son of sevenless

T2D	Type 2 diabetes
TBE	Tris-borate-EDTA buffer
TBS	Tris- buffered saline
TBST-T	TBS with 0.1% Tween-20
TGF-α	Transforming growth factor-α
TGF-β	Transforming growth factor-β
TNF-α	Tumour necrosis factor-α
VCAM-1	Vascular cell adhesion molecule-1
VEGF –A/B/C/D/E	Vascular endothelial growth factor–A/B/C/D/E
VEGFR-1/2/3	Vascular endothelial growth factor receptor-1/2/3
VHL	Von Hippel-Lindau
VSMCs	Vascular smooth muscle cells
vWf von-	Willebrand factor
Wb	Westernblotting

Chapter 1 - Introduction

1.1 Neovascularisation

Vascular endothelial cells form the integral and fundamental constituents of the vascular system for growth and survival. Endothelial cells (ECs) line the inner surface of the blood vessels. Neovascularization is an umbrella (or broad) term used to represent physiologically complex processes, namely, vasculogenesis, angiogenesis and arteriogenesis (Figure1.1). Vasculogenesis is a de novo process solely used to describe the process of blood vessel formation in the embryo. In vasculogenesis, undifferentiated endothelial progenitor cells (EPCs) migrate to the sites of new vessel growth and then follow on to differentiate into mature ECs (Flamme, Frolich et al. 1997). The process is completed by the formation of the vascular plexus (Gerhardt and Betsholtz 2003). Recent studies are now asserting that this process contributes to the adult blood vessel formation as well (Drake 2003, Eguchi, Masuda et al. 2007). Angiogenesis is the development of new microvessels from pre-existing vasculature, involving proliferation and migration of the ECs; in short, it is the growth of new blood vessels from pre-existing ones. The processes of angiogenesis and vasculogenesis are distinct. Despite the differences in the molecular and cellular levels governing the sequence of events in both of these processes, an overlap of several genes that are essential for angiogenesis are likewise vital for vasculogenesis as well (Folkman and D'Amore 1996, Risau 1997). The new vessels formed through angiogenesis can either develop via sprouting angiogenesis, or non-sprouting (intussusceptive) microvascular growth (IMG) (Ribatti 2006). Sprouting angiogenesis is an important and common mechanism of vessel growth coordinated by sequential steps (Ribatti and Crivellato 2012). Non-sprouting angiogenesis occurs by intussusceptions and vessel elongation from the pre-existing vessel growth. This development is achieved either by the merging of the pre-existing capillaries or by the fusion of additional ECs into the already existing vessels, consequently increasing the length and the diameter of

the tubular structure as well. Non-sprouting angiogenesis was shown to take place in the myocardium, skeletal muscle and during the process of wound healing (Patan, Haenni et al. 1996, Djonov, Baum et al. 2003). Overall, both sprouting and non-sprouting angiogenesis occur in the heart, lung and yolk sac (Risau 1997) (Figure 1.2).

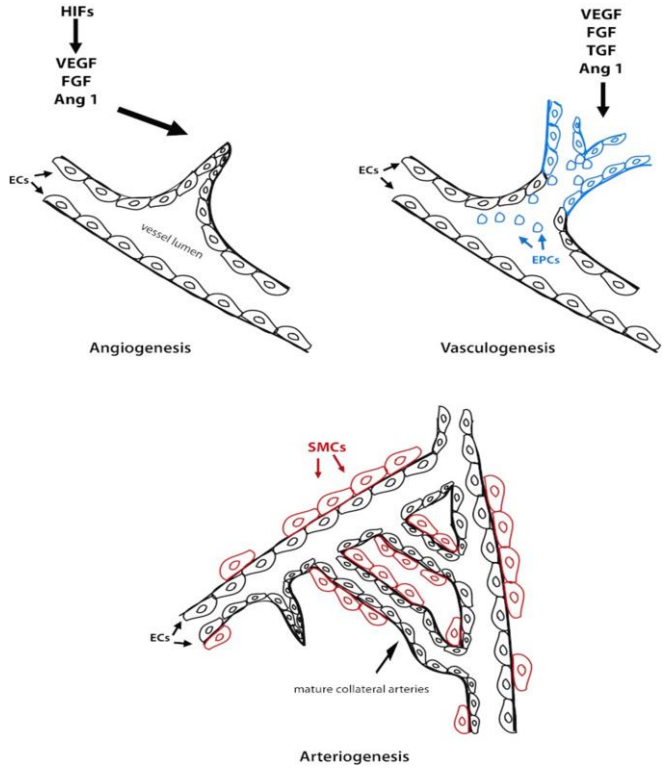


Figure 1.1 Neovascularisation - The processes of angiogenesis, vasculogenesis, and arteriogenesis. *Angiogenesis, new microvessels generated from pre-existing vasculature by the proliferation and migration of mature ECs in response to agonists of angiogenesis, i.e growth factors. In vasculogenesis undifferentiated EPCs circulate to the sites of new vessel growth, where they differentiate into mature ECs. Arteriogenesis promotes neovascularisation via migration and proliferation of ECs and SMCs forming mature collateral arteries. Abbreviations: Ang 1, angiopoietin-1; EC, endothelial cell; EPC, endothelial progenitor cell; FGF, fibroblast growth factor; HIF, hypoxia-inducible factor; SMC, smooth muscle cell; VEGF, vascular endothelial growth factor (Szoke and Brinchmann 2012).*

Arteriogenesis involves the migration and proliferation of two major cell types; ECs and smooth muscle cells (SMCs). During this process, the expansive growth of the collateral arteries from the sprouting of pre-existing vessels form collateral bridges between the arterial networks in response to endogenous growth factors and cytokines, such as VEGF, FGF, TGF, Ang 1, which are released secondary to tissue ischemic event.

1.2 Physiological Angiogenesis

Physiological angiogenesis is fundamental for embryonic development and is characterised by tight regulation, both spatially and temporally. Angiogenesis is essential for a healthy adult to maintain functions of the female reproductive system, wound and fracture healing, and tissue repair.

1.2.1 Angiogenesis: A Multistep Process

Angiogenesis is a complex process involving extensive interaction between cells, soluble factors and extracellular matrix [ECM] components. The formation of a vascular network, subsequent to angiogenesis, requires fine coordination of sequential events regulated by antagonistic factors to stimulate and inhibit angiogenesis (Risau 1997). Metabolic demands regulate the vascularisation of tissues as well as tumours. The tissue diffusion limit of oxygen ranges between 100-200 μm, indicating hypoxia to be the most significant pro-angiogenic trigger (Schaafhausen, Yang et al. 2013). Hypoxia induces the regulation of hypoxia-inducible factor (HIF) protein signalling cascade (Semenza 2001), which in turn up-regulates the endogenous release of various growth factors and cytokines, such as VEGF, fibroblast growth factor-2 (FGF-2) and placenta growth factor (PlGF) to name a few. These growth

factors and cytokines diffuse into the surrounding environment, bind to their specific receptors and activate the ECs lining the pre-existing blood vessels. The activated endothelial cells loosen their cell contacts and release proteases such as matrix metalloproteinases [MMPs], resulting in the degradation of the base membrane surrounding the pre-existing vessel. ECs migrate towards angiogenic stimuli into the interstitial spaces. Angiogenesis likewise relies on the adhesion receptor integrins such as αvβ3 and αvβ5 that facilitate EC migration through their interaction with a variety of ECM components (Cheresh 1991, Leavesley, Schwartz et al. 1993). Besides being a fundamental factor for the survival of the nascent vessels, via regulating cell adhesion to the matrix, αvβ3 associates in the activation of vascular endothelial growth factor receptor 2 (VEGFR-2) triggered by its ligand VEGF (Soldi, Mitola et al. 1999, Borges, Jan et al. 2000). Sprouting ECs proliferate, forming a new lumen, and the generation of new basement membrane takes place. These ECs that deposit on the new basement membrane, secrete growth factors such as platelet-derived growth factor (PDGF), which recruit pericytes in small vessels and SMCs in larger vessels for the structural support and vascular integrity of the vessel (Carmeliet 2000).

Angiogenesis is a highly complex process involving the concerted actions of several other factors, such as the angiopoietins and ephrins which regulate vessel stability upon activation of their specific receptors (Yancopoulos, Davis et al. 2000). PDGF and the angiopoietins complement this process at the later stages of angiogenesis, bridging communications between the ECs and the mesenchyme in the surrounding areas (Hammes, Lin et al. 2002, Guo, Hu et al. 2003). Finally, the fusion of the newly formed vessels initiates the flow of the blood.

Key Stage	Markers
Stage One: Endothelial cell activation in response to angiogenic factors.	**Basic Fibroblast Growth Factor (bFGF):** a potent stimulatory factor for endothelial cell migration and proliferation. **Vascular Endothelial Growth Factor (VEGF):** initiates cell proliferation and migration.
Stage Two: Degradation of the capillary wall by extracellular proteinases.	**Matrix Metalloproteinases (MMPs):** MMP1 (a collagenase) and MMP2 are expressed during angiogenesis and act to degrade extracellular matrix components.
Stage Three: Formation of a branch point in the vessel wall.	**Integrins:** expressed on newly forming vessels.
Stage Four: Migration of endothelial cells into the extracellular matrix towards the angiogenic stimulus.	**Integrins:** allow migrating endothelial cells to interact with specific components of the surrounding matrix. **MMPs and urokinase:** aid migration of endothelial cells into the surrounding matrix.
Stage Five: Re-organisation of endothelial cells to form tubules with a central lumen.	**Angiopoietin (Ang 1):** produced by surrounding stromal cells; facilitates endothelial cell survival and stabilisation of new capillary tubes.
Stage Six: Interconnection of the new tubules to form a network (anastomosis).	**Platelet Derived Growth Factor (PDGF):** produced by endothelial cells of the new capillaries; recruits pericytes which stabilize the new vessels.

Table 1.1 Schematic representation of the key stages in angiogenesis and the relevant growth factors involved at each stage. *[http://www.cellworks.co.uk/angiogenesis.php].*

The sequential events involved in vasculogenesis leading to mature circulatory system via angiogenesis are tightly regulated by several endogenously released growth factors and cytokines pictorially illustrated in Figure 1.2.

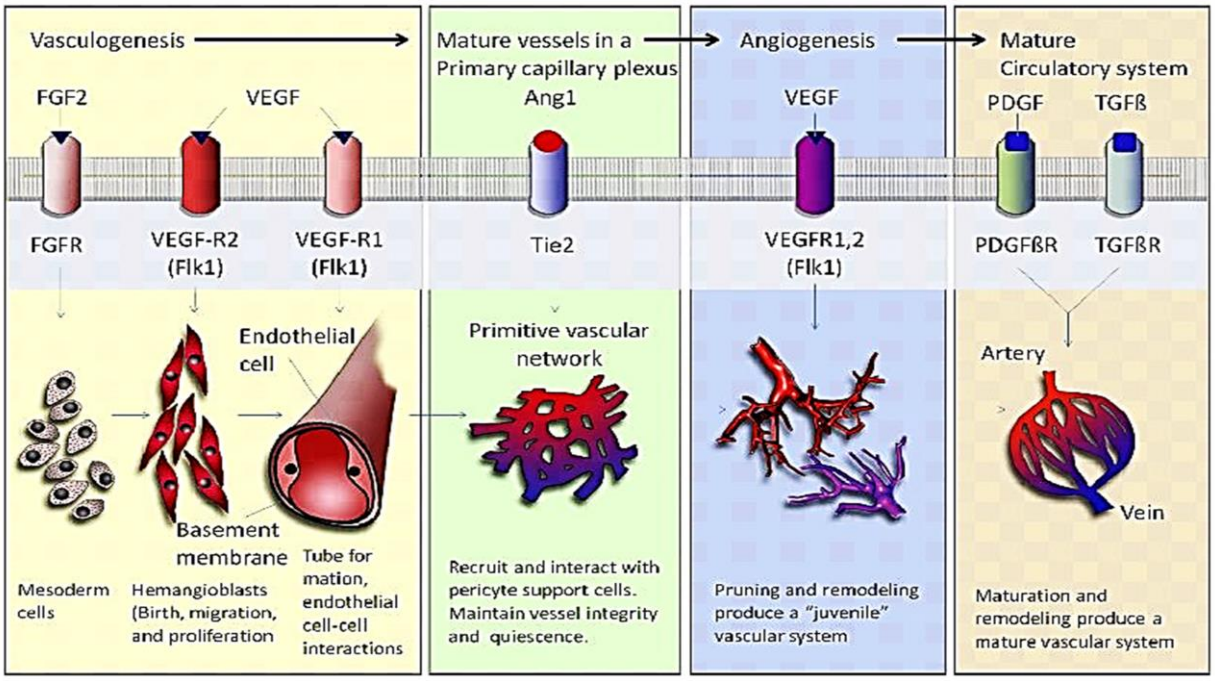


Figure 1.2 Pictorial illustration representing the sequential events involved in vasculogenesis and angiogenesis *(Chai 2013)**.***

1.2.2 Angiogenesis in female reproductive system

Angiogenesis is a fundamental process underlying the action and function of the female reproductive system; notably the menstrual cycle and the establishment of pregnancy. Rapid cyclic growth and shedding of the endometrium take place throughout the reproductive life of a female. The process involving the development and function of corpus luteum is largely dependent on the growth of new blood vessels (Klauber, Rohan et al. 1997). EC proliferation occurs throughout the menstrual cycle, reflecting the continual vascular remodelling occurring in the endometrium (Rogers, Donoghue et al. 2009). Studies have demonstrated that VEGF, PlGF, FGF-2 and other angiogenesis promoting factors are expressed in human endometrium, decidua and placenta (Rogers, Donoghue et al. 2009). Endometrial angiogenesis predominantly occurs in the basalis layer, and in the sub epithelial capillary plexus of the functionalis layer during the phase of proliferation and early secretion of the menstrual cycle. Two distinct peaks of EC migratory activity that correspond to the cyclic changes in EC proliferation are apparent in the functionalis region of the endometrium. These occur during the post menstrual and mid to late proliferative phases of the cycle (Rogers, Lederman et al. 1998). Despite the certainty of angiogenesis in the endometrium during the menstrual cycle, reports from several studies in animal models have failed to establish a correlation between the factors triggering the angiogenic switch and neovascularisation in the endometrium (Weston and Rogers 2000).

Studies exploring the type of angiogenesis occurring in the endometrium reported that the expression of αvβ3 integrin [expressed by ECs during angiogenesis] localised in the ECs within the existing vessels and not detected in the vascular sprouts. This observation strongly suggests intussusceptions and vessel elongation as the major mechanism of angiogenesis in the endometrium (Rogers, Lederman et al. 1998). The uterus is home to circulating

endothelial progenitor cells [EPCs] that differentiate into ECs (Asahara, Masuda et al. 1999). Dr Shunichiro Miyoshi and the group recently reported how multipotent stem cells derived from the menstrual blood exhibited stem cells properties. These precursor cells, when co-cultured with rat cardiomyocytes, formed sheets of heart muscle tissue (Hida, Nishiyama et al. 2008).

Several molecules are involved as mediators in endometrial angiogenesis, including VEGF, PlGF, Ang-1 and Ang-2 (Maisonpierre, Suri et al. 1997) and FGF-2 (Gospodarowicz, Cheng et al. 1985, Ferrara, Chen et al. 1998, Demir, Yaba et al. 2010). It was reported that a clear association exists between the expression of VEGF and angiogenesis occurring in the endometrium (Graubert, Ortega et al. 2001). In 1998 Ferrara and group, reported that rats treated with soluble receptor VEGFR-1 (sVEGFR-1) demonstrated complete suppression of corpus luteum angiogenesis (Ferrara, Chen et al. 1998). VEGF expression has also been correlated with angiogenesis regulated by oestrogen (Shweiki, Itin et al. 1992). Moreover, it has been found that the regulatory regions of the VEGF gene harbour oestrogen response elements (Mueller, Vigne et al. 2000). The majority of endometrial VEGF appears to be secreted by the glandular epithelium when compared to the stromal cells (Nayak, Critchley et al. 2000, Bussolati, Dunk et al. 2001). During days 1-3 of the menstrual cycle (Nayak, Critchley et al. 2000) maximal expression of VEGF induced by hypoxia following the constriction of spiral arterioles in conjunction with the expression of transforming growth factor (TGF) α and interleukin (IL)-1β was noticed (Varma and Mascarenhas 2001). Collectively, the studies shed light on the multiple factors involved in the tight regultion of endometrial angiogenesis.

1.2.3 Wound Healing

New vessel formation is critical for the process of wound healing. Capillary regression followed by the successful healing of the wound and resultant termination of the process takes place. Neovascularisation into the wound is fundamental for the provision of oxygen and nutrients to the tissue during repair, to promote granulation tissue formation (Schafer, Maier et al. 1994) and to clear debris from the wound (Dvorak, Nagy et al. 1988). Neovascularisation for the process of wound healing depends on several factors, such as cell to cell interactions, cell and ECM interactions, and most importantly the balance between agonist and antagonist that have a direct effect on the "angiogenic switch". The angiogenic switch refers to the time restricted event, wherein the balance between the pro- and anti-angiogenic mediators tilts, resulting in unwanted or unstable cellular events. Tissue injury is followed by plasma exudation, including the degranulation of plasma fibrinogen. Plasma fibrinogen then acts as a substrate and generates fibrin-containing matrix, which eventually is substituted by the granulation tissue (Schafer, Maier et al. 1994, To and Midwood 2011). Degranulation of platelets and release of growth factors are the initial events at the wound site (Maloney, Silliman et al. 1998). Granulation involves the process of proteolysis by plasmin, which is produced by plasminogen in the interstitial fluid and plasma (Miyashita, Wenzel et al. 1988). Recruitment of polymorphonuclear leukocytes [PMNs], monocytes, fibroblasts, and capillary endothelial cells organise the primary wound matrix (Dvorak, Nagy et al. 1988). Neovascularisation, which plays a critical role in successful wound healing, is predominantly regulated by the growth factors FGF-2 (Ortega, Ittmann et al. 1998), VEGF (Nissen, Polverini et al. 1998) and PlGF (Carmeliet, Moons et al. 2001). These growth factors are released by both endothelial and non-endothelial cells in response to inflammatory mediators. Macrophages play a significant role in contributing to the healing process by releasing potent

angiogenic factors (Swift, Kleinman et al. 1999). During wound healing, PlGF released by vascular and non-vascular cells is known to have an autocrine effect on ECs (Migdal, Huppertz et al. 1998, Dewerchin and Carmeliet 2012) as well as a role in recruiting macrophages to the site of injury. These inflammatory mediators further release both angiogenic and lymphangiogenic (formation of lymphatic vessels from pre-existing lymphatic vessels) molecules, in addition to proinflammatory cytochemokines (Selvaraj, Giri et al. 2003, Dewerchin and Carmeliet 2012) contributing to the process of wound healing.

1.3 Pathological Angiogenesis

Pathological angiogenesis occurs when the dynamic balance between factors that induce the formation of blood vessels and those that inhibit the process is obliterated. A distinct margin lies between physiological and pathological settings (Folkman 1995). Although many positive and negative regulators function in both situations, EC proliferation is firmly controlled in the former, whereas in the latter, the unchecked growth of blood vessels lead to several diseases in different tissues, some of which illustrated in Figure 1.3

In both normal and pathological angiogenesis, hypoxia is the main driver of the angiogenic process via the hypoxia-inducible factor (HIF-1) receptor. Furthermore, growth factors, hormones and inflammatory mediators enhance the expression of angiogenic factors, such as VEGF, PlGF, FGF and respective receptors, thereby switching on the process of unchecked angiogenesis (Galzie, Kinsella et al. 1997, Jiang, Agani et al. 1997, Carmeliet, Dor et al. 1998, Carmeliet 2005). The remarkable ability of the ECs to divide excessively results in excessive angiogenesis, which can lead to pathologies of inflammatory, ocular and malignancy disorders (Carmeliet 2005). Similarly, insufficient angiogenesis can lead to EC

dysfunction, vessel regression or malformation, contribute to preeclampsia (PE), ischemic heart disease, peripheral vascular disease, and ulcers to name a few (Folkman 1995, Carmeliet 2005).

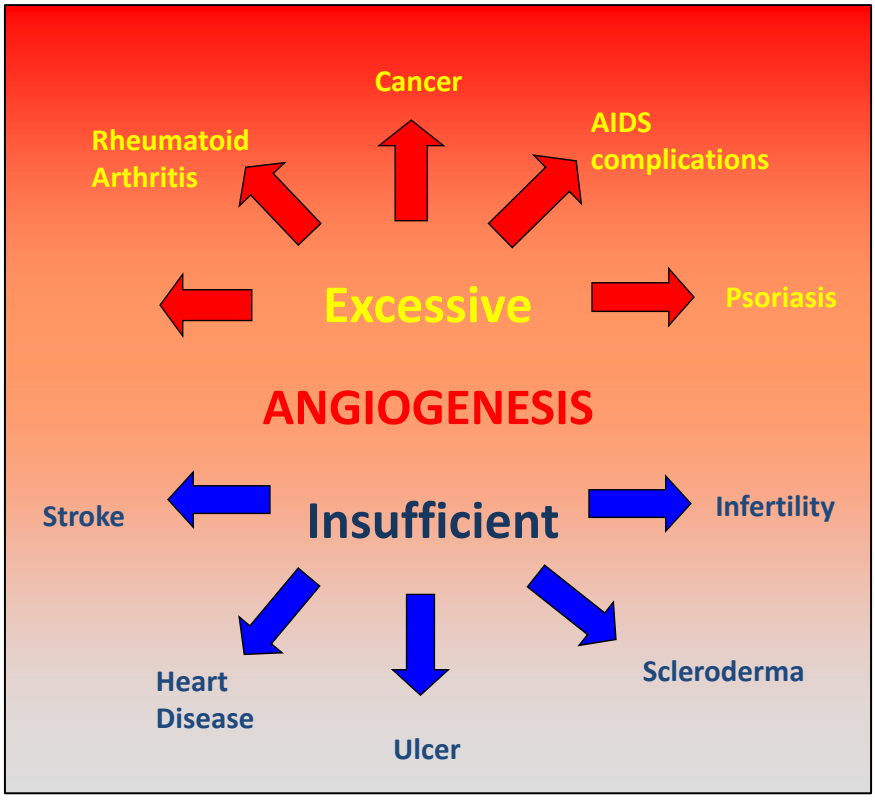


Figure 1.3 Angiogenesis off-balance common denominator for pathologies. *Schematic representation of excessive angiogenesis leading to inflammatory and/or malignant disorders and insufficient angiogenesis giving rise to cardiovascular disease, peripheral vascular disease and other pathologies.*

1.4 Role of growth factors in angiogenesis

VEGF-mediated angiogenesis is critical for organ formation during embryogenesis. Angiogenesis is tightly regulated by a variety of pro- and anti-angiogenic factors involved in the stimulation of angiogenesis. The imbalance between pro- and anti-angiogenic factors is manifested in pathological conditions, including cardiovascular disorders, tumours and inflammatory disorders. Hypoxia-driven up-regulation of pro-angiogenic compounds,

inflammatory cytokine-derived signals, or mutations leads to pathological angiogenesis (Malhotra 1989, Velasco 2002, Streit 2003, Yano 2003). A range of pro-angiogenic factors, including angiopoietins, FGF, transforming growth factor-β (TGF-β), and the members of the VEGF family such as PlGF, play key roles in the regulation of angiogenesis as well as lymphangiogenesis (Folkman 1987, Keck 1989, Yang 1990, Suri 1996).

1.5 Factors regulating angiogenesis

1.5.1 Vascular Endothelial Growth Factor [VEGF]

VEGF was initially described as a vascular permeability factor (Brown, Weiss et al. 1980, Senger, Galli et al. 1983) that exerted its effects on non-angiogenesis related cellular functions as well. The identification of VEGF [or VEGF-A] has revolutionised the understanding of angiogenesis and of the prominent role it holds in physiological and pathological events. VEGF is a member of the PDGF family of growth factors. VEGF and PlGF (Maglione, Guerriero et al. 1991) are two prominent members of the VEGF family of secreted proteins that exert endothelial and non-endothelial cellular mechanisms, along with the others members VEGF-B (Olofsson, Pajusola et al. 1996), VEGF-C (Joukov, Pajusola et al. 1996, Lee, Gray et al. 1996), VEGF-D (Achen, Jeltsch et al. 1998), VEGF-E (Ogawa, Oku et al. 1998, Meyer, Clauss et al. 1999) and VEGF-F (Komori, Nikai et al. 1999). The members of the VEGF family are structurally related dimeric glycoproteins with different physical and biochemical characteristics, functional via three receptor tyrosine kinases (RTKs). VEGF displays the ability to stimulate ECs to proliferate, migrate and survive in serum-deprived conditions (Breen 2007).

VEGF-B, which is also known as VEGF-related factor [VRF], has its gene located on chromosome 11q13 (Paavonen, Horelli-Kuitunen et al. 1996). VEGF-B is expressed early in

foetal development and is distributed in different types of tissues, specifically in skeletal muscle and heart (Olofsson, Pajusola et al. 1996, Aase, Lymboussaki et al. 1999). VEGF-B binds to VEGFR-1 [fms-like tyrosine kinase 1/Flt-1], but not VEGFR-2 [kinase domain receptor - KDR/ Flk-1] or VEGFR-3 (Olofsson, Korpelainen et al. 1998). Two splice variant isoforms VEGF-B_{167} and VEGF-B_{186} are produced (Olofsson, Pajusola et al. 1996), which bind to heparin and neuropilin-1 respectively (Makinen, Olofsson et al. 1999).

VEGF-C and VEGF-D act as lymphangiogenic growth factors (Joukov, Pajusola et al. 1996, Achen, Jeltsch et al. 1998). VEGF sub-types VEGF-C and D are proteolytically processed; no splice variants have been reported in humans yet (Baldwin, Roufail et al. 2001). However, two different protein isoforms with distinct C-termini, VEGF-D_{358} and VEGF-$D_{326,}$ with binding capacity for VEGFR-2 and VEGFR-3 have been identified in mice (Baldwin, Catimel et al. 2001)

VEGF-E and VEGF-F are non-mammalian proteins related to VEGF. VEGF-E has been identified in the Parapoxvirus genome (Lyttle, Fraser et al. 1994). VEGF-E was identified in the genome of various strains of the Orf virus [NZ-2, NZ-7 and D1701], a member of the Poxvirus superfamily (Lyttle, Fraser et al. 1994, Ogawa, Oku et al. 1998, Meyer, Clauss et al. 1999, Wise, Veikkola et al. 1999). Lesions caused by this virus are highly vascularised (Larcher, Murillas et al. 1998). Despite low sequence similarity to VEGF-A [16-20 % apparent homology], VEGF-E induces comparable levels of cell proliferation and permeability to that of VEGF-A_{165} (Ogawa, Oku et al. 1998, Meyer, Clauss et al. 1999, Wise, Veikkola et al. 1999). VEGF-E exerts these cellular activities via VEGFR-2. It was hypothesised that VEGF-E may have a phylogenetic origin in the vertebrate genome (Ogawa, Oku et al. 1998).

VEGF-F is the seventh member of the VEGF family was discovered in recent times. VEGF-F was identified in the venom of the snake (viper) (Yamazaki, Takani et al. 2003). VEGF-F yields two designated proteins: vammin of 110 residues and VR-1 with 109 residues (Otrock, Makarem et al. 2007). VEGF-F shares 50% homology with $VEGF_{165}$ and selectively binds to VEGFR-2 (Otrock, Makarem et al. 2007).

Structure and Functional Properties

VEGF is a basic, heparin-binding, homodimeric glycoprotein of 45,000 Daltons (Ferrara, Houck et al. 1992) expressed in different tissues, including the brain, kidney, liver and spleen, and by many other cell types (Veikkola and Alitalo 1999). VEGF shares about 20% homology of amino acid sequence with PDGF-A and PDGF-B chains (Keck, Hauser et al. 1989, Tischer, Gospodarowicz et al. 1989, Ferrara, Houck et al. 1992).

VEGF, a.k.a VEGF-A has received greater attention and is a key mitogen regulating both physiological and pathological angiogenesis. VEGF is expressed in nine different isoforms, $VEGF\text{-}A_{121}$, $VEGF\text{-}A_{145}$, $VEGF\text{-}A_{148}$, $VEGF\text{-}A_{162}$, $VEGF\text{-}A_{165}$, $VEGF\text{-}A_{165b}$, $VEGF\text{-}A_{183}$, $VEGF\text{-}A_{189}$ and $VEGF\text{-}A_{206}$ generated due to alternative mRNA splicing (Tischer, Mitchell et al. 1991, Ferrara, Houck et al. 1992, Bates, Cui et al. 2002) [see Figure 1.3]. The human VEGF gene is located on chromosome 6p21.3 composed of eight exons (Vincenti, Cassano et al. 1996). The *N*-terminal of VEGFR-binding domain is the common region to all the isoforms which is encoded on exons 1-5 (Tischer, Mitchell et al. 1991). $VEGF\text{-}A_{165}$ lacks exon six coding regions, physiologically is the most abundant isoform of VEGF-A (Tischer, Mitchell et al. 1991) and binds to both VEGFR-1 and VEGFR-2. However, the binding

affinity of VEGF-A_{165} to VEGFR-1 is ten-fold higher than VEGFR-2 (de Vries, Escobedo et al. 1992, Terman, Dougher-Vermazen et al. 1992, Quinn, Peters et al. 1993). A new group of VEGF-A isoforms generated by the alternative splicing of the distal site at exon eight site were identified. Though these isoforms are similar in length to the classic VEGF isoforms, they differ in their C-terminal six amino acids. These new isoforms were designated the name $VEGF_{xxx}b$, wherein xxx denoted the amino acid number of the mature protein. Currently, two isoforms have so far been identified and assigned as VEGF-$A_{165}b$ and VEGF-$A_{159}b$. $VEGF_{xxx}b$ were reported to inhibit pro-angiogenic functions of VEGF (Bates, Cui et al. 2002). The structural variation and the distinct binding patterns of the VEGF subtypes to the receptors are illustrated in figure 1.4.

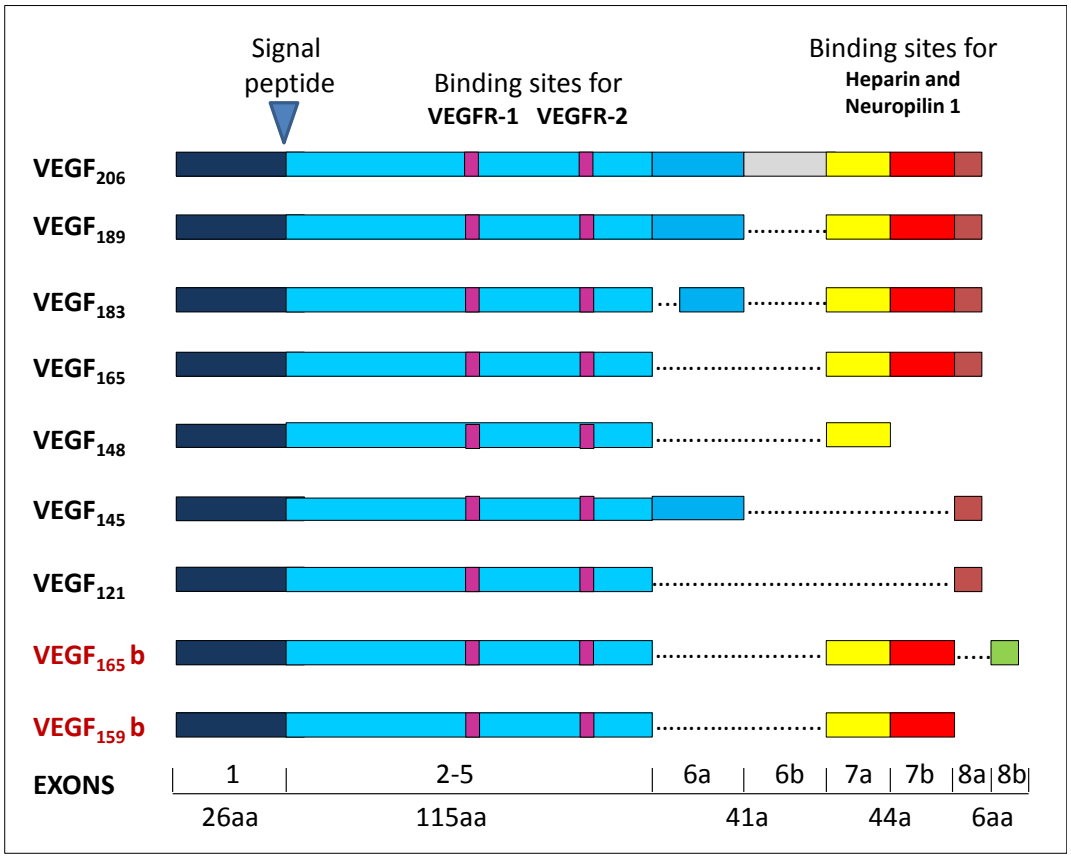


Figure 1.4 Exonic structure of the VEGF gene and the identified splice variants of VEGF-A. *Schematic representation of the selected VEGF-A variants (Grunewald, Prota et al. 2010).*

Receptor Binding

The VEGF ligands bind to their specific receptors in a distinct pattern, as illustrated in Figure 1.5. Each of the VEGF family members selectively bind to the VEGFR-1, 2 and 3 RTKs and neuropilin-1, 2 co-receptors, to manifest the diversity of their biological functions (Terman, Khandke et al. 1994, Makinen, Olofsson et al. 1999, Veikkola and Alitalo 1999, Breen 2007, Grunewald, Prota et al. 2010). VEGF isoforms bind to the cell surface and ECM by interacting with heparin sulphate proteoglycans [HSPG] that modulate the interactions of VEGFs and their receptors. The alternative splice variants of VEGF-A; VEGF-A_{121}, VEGF-$A_{145,}$ VEGF-$A_{165,}$ VEGF-A_{189} and VEGF-A_{206} have been shown to bind to both VEGFR-1 and VEGFR-2 (de Vries, Escobedo et al. 1992, Neufeld, Cohen et al. 1996). VEGF-A_{121} is a highly diffusible weak acidic protein that lacks exons 6 and 7; it neither binds to heparin nor neuropilin. VEGF-A_{189} and VEGF-A_{206} are retained in the extracellular matrix [ECM] and contain residues encoded by 6 and 7. These subtypes bind to heparin containing proteoglycans with high affinity (Houck, Leung et al. 1992). The C-terminal region of VEGF-A_{165} is critical for the heparin-binding and mitogenic activities. Exogenous heparin potently inhibited the binding of VEGFR-1, with both VEGF-A_{165} that binds to immobilised heparin, and VEGF-A121, a splice variant lacking heparin binding affinity (Cohen, Gitay-Goren et al. 1995).

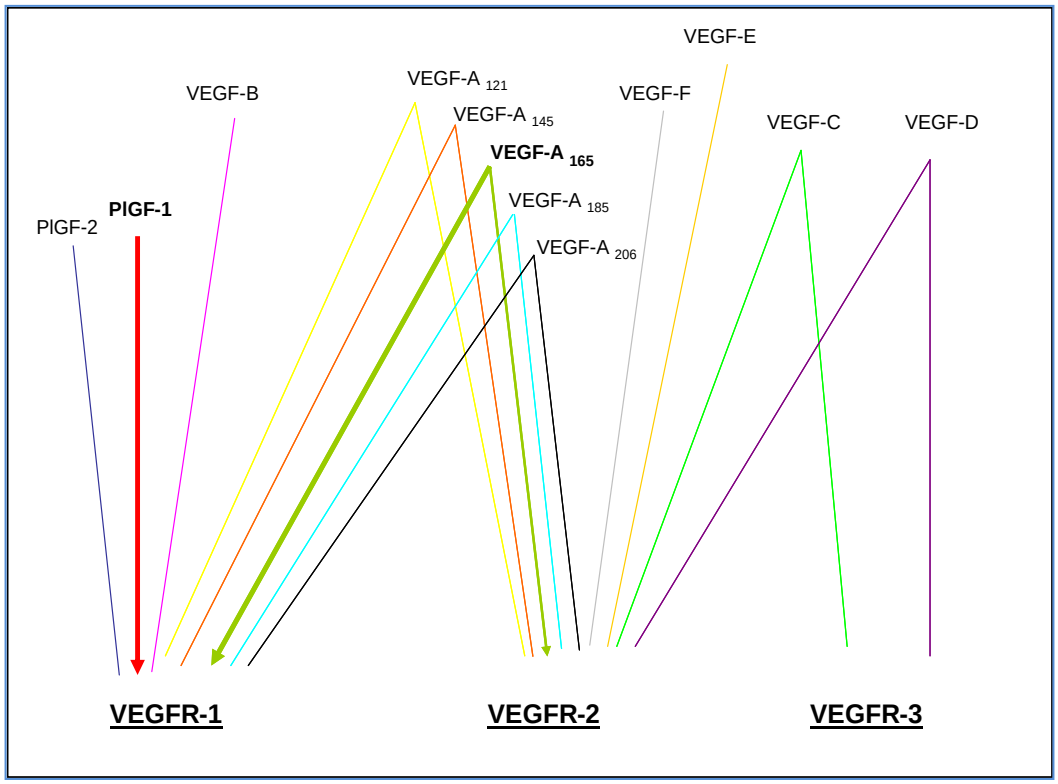


Figure 1.5 Schematic representation of VEGF family/subtype ligand-receptor interactions

Expression and regulation

VEGF is a key regulator of vasculogenesis and angiogenesis (Plate and Warnke 1997, Josko, Gwozdz et al. 2000) and is produced by ECs, macrophages, fibroblasts and smooth muscle cells (Plate and Warnke 1997). Both *in vitro* and *in vivo* studies reported that VEGF binds to the specific receptors on ECs and act as a direct inducer of angiogenesis (Risau 1997). The two best characterised VEGF receptors, VEGFR-1 and VEGFR-2, are specific RTKs that together with PDGF receptors form the subtype III of RTK receptors through which VEGF executes cellular activities (Shibuya, Yamaguchi et al. 1990, de Vries, Escobedo et al. 1992, Terman, Dougher-Vermazen et al. 1992, Josko, Gwozdz et al. 2000). Although VEGFR-1

binds to VEGF with substantially higher affinity, most of the biological effects of VEGF seem to be VEGFR-2-mediated (Holash, Davis et al. 2002) on ECs. Naturally occurring splice variant short soluble protein [sVEGFR-1] of VEGFR-1 retains its high-affinity binding to VEGF. sVEGFR-1 is a negative regulator effecting VEGF availability or may prolong the different VEGF-induced EC activities (Kendall and Thomas 1993, Plate and Warnke 1997, Hornig, Barleon et al. 2000, Hasan and Jayson 2001).

VEGF-A activation of phosphatidylinositol-3 kinase (PI3K) and Bcl-2 signalling pathways promotes EC survival by inducing the expression of anti-apoptotic proteins Bcl-2 (Gerber, Dixit et al. 1998). VEGF-A mRNA expression is up-regulated by growth factors, inflammatory cytokines and hormones (Bates and Harper 2002), with the prime factor being hypoxia. Several growth factors like FGF-2 also regulate VEGF mRNA expression in a paracrine and autocrine manner, independent of hypoxic microenvironment (Seghezzi, Patel et al. 1998). It is now recognised that Hypoxia-inducible factor-1 alpha [HIF-1α] is a key mediator of the hypoxic responses. HIF-1α is involved in all vital physiological and pathological processes. Hypoxia-induced VEGF regulation elicits physiological responses via multiple molecular mechanisms. HIF-1 participates in essential developmental and physiological processes, including angiogenesis, energy metabolism, iron homeostasis, cell proliferation and viability via transactivation of target genes (Heme Oxygenase 1, Nitric Oxide Synthase 2, Plasminogen Activator Inhibitor 1, VEGF, VEGFR-1, Insulin-like Growth Factor 2 (IGF-2), Transforming Growth Factor 3 (TGF3), etc.) (Mukhopadhyay, Mazumder et al. 2000, Semenza 2000). Additionally, HIF-1 transactivation of target genes leads to either protective or pathologic responses in several major disease states, such as myocardial ischemia (MI), preeclampsia (PE), intrauterine foetal growth restriction (IUGR), pulmonary

hypertension, cerebral ischemia and cancer (Semenza 2000). The role of von Hippel–Lindau [VHL], tumour suppressor gene in HIF-1α dependent hypoxic responses, has also been described (Tammela, Enholm et al. 2005). Mutations in the VHL gene are allied with increased angiogenesis and tumours with VHL mutations that display increased VEGF-A expression. VHL targets the degradation of HIF subunits; consequently loss of VHL increases HIF1α levels (Ivan, Kondo et al. 2001, Jaakkola, Mole et al. 2001). In hypoxic situations, HIF-mediated regulation involved PI3K/Akt signalling mechanism (Semenza 2000) modulating VEGF-A expression.

Biological activity & Role

VEGF is very critical for the differentiation of ECs and morphogenesis of the vascular system during development. During embryogenesis loss of a single *vegf* allele resulted in the substantially deficient vascular network, leading to the death of mice (Carmeliet, Ferreira et al. 1996, Ferrara, Carver-Moore et al. 1996). Embryonic lethality is also observed in mice following a two-fold over-expression of VEGF, restating the significance of *vegf* gene dosage during development (Miquerol, Langille et al. 2000).

As a multifunctional cytokine, VEGF is a potent stimulator of angiogenesis *in vivo*, and growth, migration, and differentiation of ECs *in vitro* (Ferrara, Carver-Moore et al. 1996, Terman and Dougher-Vermazen 1996, Shibuya, Luo et al. 1999). VEGF also elicits an angiogenic response in a variety of *in vivo* models including the chick chorioallantoic membrane (Leung, Cachianes et al. 1989, Plouet, Schilling et al. 1989), the rabbit cornea (Ferrara and Davis-Smyth 1997), the primate iris, and the rabbit bone (Ferrara and Davis-Smyth 1997). VEGF is named as a key regulator of pathological growth of the blood vessels

in many conditions including, inflammation, retinopathies, psoriasis and arthritis (Hicklin and Ellis 2005, Dvorak 2006, Folkman 2007).

The avb3 integrin, an adhesion receptor for extracellular matrix components with an exposed Arginine-glycine-aspartic acid (RGD) sequence is an attractive target for anti-angiogenic therapy since it is exclusively present on the cell surface of activated ECs but absent in quiescent endothelium or other cell types (Eliceiri and Cheresh 2000). Promising results with gene therapy have been obtained in therapeutic angiogenesis. Intramuscular gene transfer of $VEGF_{165}$ and intramyocardial administration of an adenoviral vector expressing the $VEGF_{121}$ cDNA improved collateral vessel development in patients with critical limb ischemia (Arveschoug and Christensen 1999) and coronary artery disease (Rosengart, Lee et al. 1999), respectively. Although VEGF improves myocardial blood flow, it produces endothelium-derived relaxing factor (EDRF)-mediated hypotension (Hariawala, Horowitz et al. 1996), a decrease in cardiac output and stroke volume, tachycardia (Yang, Bunting et al. 2000) and, in certain studies, the formation of intramural vascular tumours at the implantation site (Lee, Springer et al. 2000). In contrast, both PlGF and FGF-2 have drawn the attention of researchers as a safer and potential therapeutic target for neovascularisation in animal models and trials (Laham, Sellke et al. 1999, Laham, Chronos et al. 2000, Lederman, Mendelsohn et al. 2002, Simons, Annex et al. 2002) (Table 1.2 and 1.3).

1.5 VEGF Receptor Tyrosine Kinases

1.5.1 VEGFR-1

VEGFR-1 was the first VEGF receptor identified and originally cloned from a human placental cDNA library (Shibuya, Yamaguchi et al. 1990). VEGFR-1 homologues were identified in other mammals including mouse, rat, goat, pig, cow, sheep, and dog (Finnerty,

Kelleher et al. 1993, Choi, Wall et al. 1994, Yamane, Seetharam et al. 1994) and VEGFR-1 gene identified in the chicken (Akimoto, Hashimoto et al. 2002) and in the zebrafish (Rottbauer, Just et al. 2005).

Structure and Function

The VEGFR-1 human gene is located on chromosome 13q12-13 (Shibuya, Yamaguchi et al. 1990), and on chromosome 5 for the mouse homologue (Rosnet, Stephenson et al. 1993). VEGFR-1 mRNA transcript ensues mainly as a 7.5-8.0 kb transcript. VEGFR-1 is composed of seven extracellular immunoglobulins [Ig] homology domains, a single transmembrane region and an intracellular tyrosine kinase domain (Shibuya, Yamaguchi et al. 1990). VEGFR-1-ligand interaction occurs via the second Ig-like loop (Davis-Smyth, Chen et al. 1996). VEGFR-1 binds to VEGF-A, VEGF-B and PlGF, with a high affinity. VEGF-B and PlGF serve as ligands specific for VEGFR-1 only (Shibuya, Yamaguchi et al. 1990, Park, Chen et al. 1994, Olofsson, Korpelainen et al. 1998).

VEGF and PlGF share a 46.3% homology; however, comparison of the crystal structures of VEGF and PlGF in association with the VEGFR-1 demonstrated an identical set of residues on PlGF and VEGF contributing towards binding to the receptor. This resemblance suggests that the biological events mediated by these two ligands may likely involve complex formation with co-receptors such as neuropilin, or heparan sulphate proteoglycans [HSPGs] (Christinger, Fuh et al. 2004).

Expression and Regulation

Endothelial cell-specific expression of VEGFR-1 occurs in human foetuses (Kaipainen, Korhonen et al. 1993) and in adult organs, VEGFR-1 mRNA exists in the quiescent endothelium (Peters, De Vries et al. 1993). VEGFR-1 is also expressed by

monocytes/macrophages and osteoblasts (Clauss, Weich et al. 1996), placental trophoblasts (Charnock-Jones, Sharkey et al. 1994, Ahmed, Dunk et al. 1997), cells of renal mesangial origin and in some hematopoietic stem cells (Zachary and Gliki 2001). VEGF and PlGF, both stimulate the production of tissue factor and chemotaxis in monocytes (Clauss, Gerlach et al. 1990) indicating that VEGFR-1 functions as a signalling receptor in this cell type. Additionally, VEGFR-1 is present on $CD34^+$ cells and has a role in reconstituting haematopoiesis after bone marrow depletion (Luttun, Tjwa et al. 2002). VEGFR-1 mRNA and protein are expressed by vascular smooth muscle cells [VSMCs]; including human brain vascular SMCs (Yang, Yao et al. 2004), human aortic SMCs, uterine myometrial SMCs (Grosskreutz, Anand-Apte et al. 1999) and bovine pericytes.

Pathologically, VEGFR-1 and its ligand PlGF were reported to be up-regulated and expressed in vessels near healing wounds, suggesting that the PlGF/VEGFR-1 alliance may perhaps play a role in the regulation of vascular permeability, vascular repair and maintenance (Peters, De Vries et al. 1993). Some melanomas and leukaemias express VEGFR-1 (Cohen, Gitay-Goren et al. 1995, Fiedler, Graeven et al. 1997, Pisacane and Risio 2005) whereas, VEGF and VEGFR-1 via autocrine and paracrine regulation are up-regulated in the vascular endothelium of numerous tumours (Shibuya 1995, Hatva, Bohling et al. 1996, Tran, Master et al. 2002). Tumour environment is hypoxic, VEGFR-1 expression (as mentioned earlier) is up-regulated by hypoxia via HIF-1α dependent mechanism, indicating direct regulation of the gene by hypoxia (Ikeda, Wakiya et al. 1996, Gerber, Condorelli et al. 1997), *in vivo* as well as *in vitro* (Tuder, Flook et al. 1995, Brogi, Schatteman et al. 1996, Li, Brown et al. 1996, Gerber, Condorelli et al. 1997, Marti and Risau 1998). Cultured pericytes, under hypoxic conditions, express VEGFR-1 (Nomura, Yamagishi et al. 1995).

Biological Effects

The VEGFR-1 cytoplasmic domain is not essential for vasculogenesis during development (Hiratsuka, Minowa et al. 1998), and very recent work demonstrated that embryos lacking VEGFR-1 displayed an increased outgrowth of ECs and hemangioblast commitment, proposing a negative role for VEGFR-1 in angiogenesis (Fong, Zhang et al. 1999). In contrast, VEGFR-1 deficient mice developed normal vessels and survived (Hiratsuka, Minowa et al. 1998). During embryonic development, VEGF-A-mediated macrophage migration was severely suppressed, in truncated membrane bound VEGFR-1 as well as its soluble isoform sVEGFR-1, suggesting a possible role for VEGFR-1 as a decoy receptor for angiogenesis during embryo development. In this manner, modulating VEGF bioavailability and leading to the prevention of EC overgrowth. VEGF-A affinity for VEGFR-1 is stronger when compared to VEGFR-2, but VEGFR-1 has a weak signal transducing activity (Park, Chen et al. 1994), that negatively regulates VEGF-induced angiogenesis by controlling its availability to VEGFR-2. VEGFR-1 is implicated in several pathologies. Anti-VEGFR-1 suppressed neovascularisation in tumours, ischemic retinae, and prevented inflammatory joint destruction in autoimmune arthritis. Since Luttun *et al.* performed VEGFR-1 studies demonstrating it to be a potentially attractive target for modulation of pathological inflammation and angiogenesis (Luttun, Tjwa et al. 2002) VEGFR-1 has drawn greater attention from researchers.

1.7 Soluble Vascular Endothelial Growth Factor Receptor-1 (sVEGFR-1)

sVEGFR-1 is a naturally occurring, short soluble protein splice variant of VEGFR-1 that mediates inhibition of VEGF, as well as PlGF interaction with their receptors (Kendall and

Thomas 1993). This soluble receptor occurs as a result of alternative splicing of VEGFR-1 mRNA and consists of only the extracellular domain of VEGFR-1. As shown in Figure 1.7, sVEGFR-1 inhibits the binding of VEGF and PlGF to their respective RTKs and prevents the cellular activities elicited by these growth factors. Additionally, sVEGFR-1 acts as a dominant-negative inhibitor blocking the formation of VEGFR-1 and -2 homo and heterodimers, inhibiting their trans-phosphorylation or activation (Kendall, Wang et al. 1996).

Levels of sVEGFR-1 are elevated in the placenta (Maynard, Min et al. 2003, Tsatsaris, Goffin et al. 2003) and serum (Zhou, McMaster et al. 2002, Koga, Osuga et al. 2003) of women with pre-eclampsia (PE). sVEGFR-1 associated with severity of the disease and sVEGFR-1 levels stated as a predominant risk factor pertaining to this disorder (Levine, Maynard et al. 2004, Thadhani, Mutter et al. 2004). PE mouse model studies reported that TNF-α will increase sVEGFR-1 (Sunderland, Thomson et al. 2011). sVEGFR-1 also inhibits wound healing, again via prevention of VEGF or PlGF interaction to their receptors by acting as a sink (Carmeliet, Moons et al. 2001, Maynard, Min et al. 2003). In summary, sVEGFR-1 can have a beneficial or harmful effect, depending on the disorder.

1.8 Placenta Growth Factor [PlGF]

1.8.1 Structure and Functional Properties

PlGF was originally identified in the placenta by Maria Graziella Perisco (Maglione, Guerriero et al. 1991). PlGF is a N-glycosylated, secretory homodimeric protein (Maglione, Guerriero et al. 1991, Iyer, Leonidas et al. 2001), belonging to the cysteine-knot super family of growth factors, encoding for a protein with an approximately 46.3% homology to VEGF in the PDGF-like amino acid sequence (Maglione, Guerriero et al. 1991, Iyer, Leonidas et al. 2001). The PlGF gene is reported to be mapped

on human chromosome 14q24-q31 (Mattei, Borg et al. 1996) and chromosome 12q in mice (DiPalma, Tucci et al. 1996). The human PlGF gene consists of seven exons (Maglione, Guerriero et al. 1993). The 3D crystal structure of PlGF illustrated in Figure 1.6.

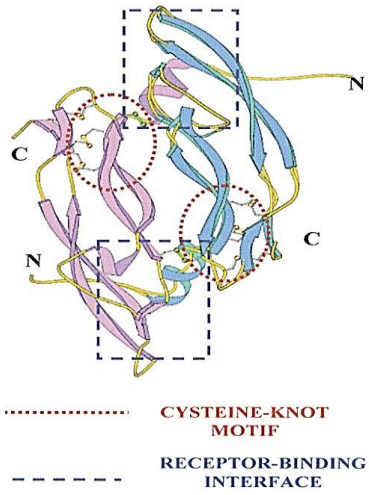


Figure 1.6 Three-dimensional crystal structure of PlGF-1. *The structure has been colour-coded to differentiate between the two subunits: Monomer A has been colour purple, and Monomer B is in cyan. The cysteine-knot motif [consists of 3-intra- and one inter-chain disulphide bridge passing through the 8-membered cysteine ring] and the receptor-binding region is highlighted at each pole of the dimer (Iyer and Acharya 2002).*

The structure of PlGF is remarkably similar to VEGF-A_{121} (Iyer and Acharya 2002). The human genome encodes four isoforms: PlGF-1 [$PlGF_{131}$], PlGF-2 [$PlGF_{152}$], PlGF-3 [$PlGF_{203}$], and PlGF-4 [$PlGF_{224}$] (Maglione, Guerriero et al. 1993, Cao, Ji et al. 1997, Yang, Ahn et al. 2003). In mice, only one PlGF mRNA encoding a 158 amino acid precursor protein has been identified that is equivalent to human PlGF-2 (Fischer, Jonckx et al. 2007, Dewerchin and Carmeliet 2012). Little is known about the difference in the functions of PlGF isoforms. PlGF-1 is composed of 131 amino acid residues per monomer, PlGF-2 consists of 170 amino acids and PlGF-3 corresponds to 216 amino acids. A schematic representation of the three splice variants of the PlGF gene is illustrated in Figure 1.7. The novel PlGF subtype

4 [PlGF-4] consists of the same sequence as PlGF-3, plus the heparin binding domain previously thought to be present only in PlGF-2 (Yang, Ahn et al. 2003). PlGF-2 can also bind to neuropilin (NRP)-1, and -2 because of an insertion of 21 basic amino acids at the carboxyl terminus (Migdal, Huppertz et al. 1998, Persico, Vincenti et al. 1999, Hoffmann, Willenborg et al. 2013). PlGF-1 and PlGF-3 are diffusible isoforms, and PlGF-2 and PlGF-4 have heparin binding domains (Hauser and Weich 1993, Persico, Vincenti et al. 1999, Yang, Ahn et al. 2003). PlGF has the natural capacity to form heterodimers with VEGF (DiSalvo, Bayne et al. 1995). Little is known about the pro-angiogenic or anti-angiogenic activity of the heterodimer VEGF: PlGF.

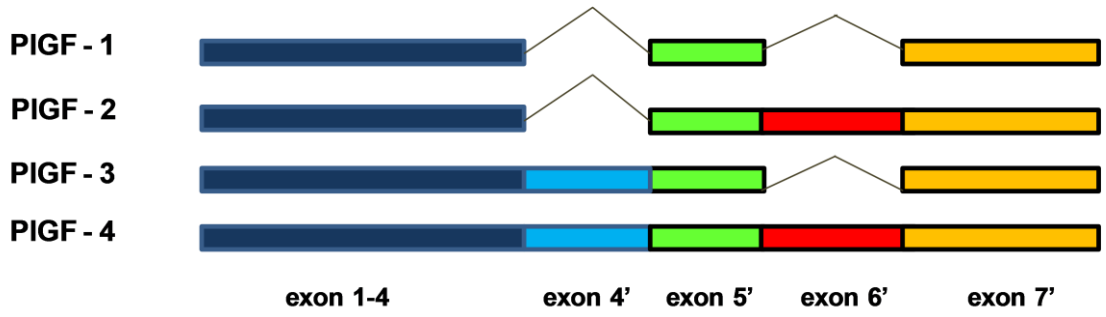


***Figure 1.7 A schematic representation of the three spliced variants of the PlGF gene**. The gene has seven exons [each colour differently]. PlGF-1 and PlGF-3 mRNAs lack exon 6. PlGF-3 mRNA has a 216 base insert between exon 4 and 5. The length of the amino acid sequence indicated for each isoform corresponds to that of the mature protein [after cleavage of the signal peptide]. (Adapted from (Takahashi and Shibuya 2005) and ethesis.helsinki.fi/.../pg/jeltsch/intro.html).*

1.8.2 PlGF and Receptor Interactions

PlGF binds to VEGFR-1, NP-1, NP-2 (Migdal, Huppertz et al. 1998, Persico, Vincenti et al. 1999) and sVEGFR-1; the natural soluble version of the receptor lacking transmembrane and intracellular domains (Kendall and Thomas 1993, Errico, Riccioni et al. 2004). One of the

glycosylated residues of the two N-linked glycosylation sites, Asn^{84}, plays a significant role in binding VEGFR-1 distinct to VEGF-A (Errico, Riccioni et al. 2004). PlGF can induce signals via VEGFR-1, independent of VEGF (Cai, Ahmad et al. 2003, Ahmad, Hewett et al. 2006).

PlGF, when bound to VEGFR-1, can indirectly promote EC proliferation via displacement of VEGF from VEGFR-1 (decoy receptor), thereby enhancing angiogenesis by liberating VEGF for activation of VEGFR-2 (Park, Chen et al. 1994). Binding of PlGF to VEGFR-1 may also induce an intermolecular cross talk between VEGFR-1: VEGFR-2 that amplifies VEGF/VEGFR-2 signalling and VEGF-driven angiogenesis (Autiero, Waltenberger et al. 2003). The schematic representation of the receptor competition and receptor cross-talk exerted by PlGF binding to VEGFR-1 illustrated in Figure 1.8.

Activation of VEGFR-1 by either PlGF or VEGF-A induces different gene expression profiles and phosphorylation of distinct tyrosine residues in the tyrosine kinase domain of VEGFR-1 (Autiero, Waltenberger et al. 2003). VEGF: PlGF heterodimers promote the formation of RTK receptors VEGFR-1: VEGFR-2 to which they preferentially bind (Autiero, Waltenberger et al. 2003), in addition to VEGFR-1: VEGFR-1. However, VEGF: PlGF heterodimers do not bind to VEFGR-2: VEGFR-2 homodimers (Eriksson, Cao et al. 2002).

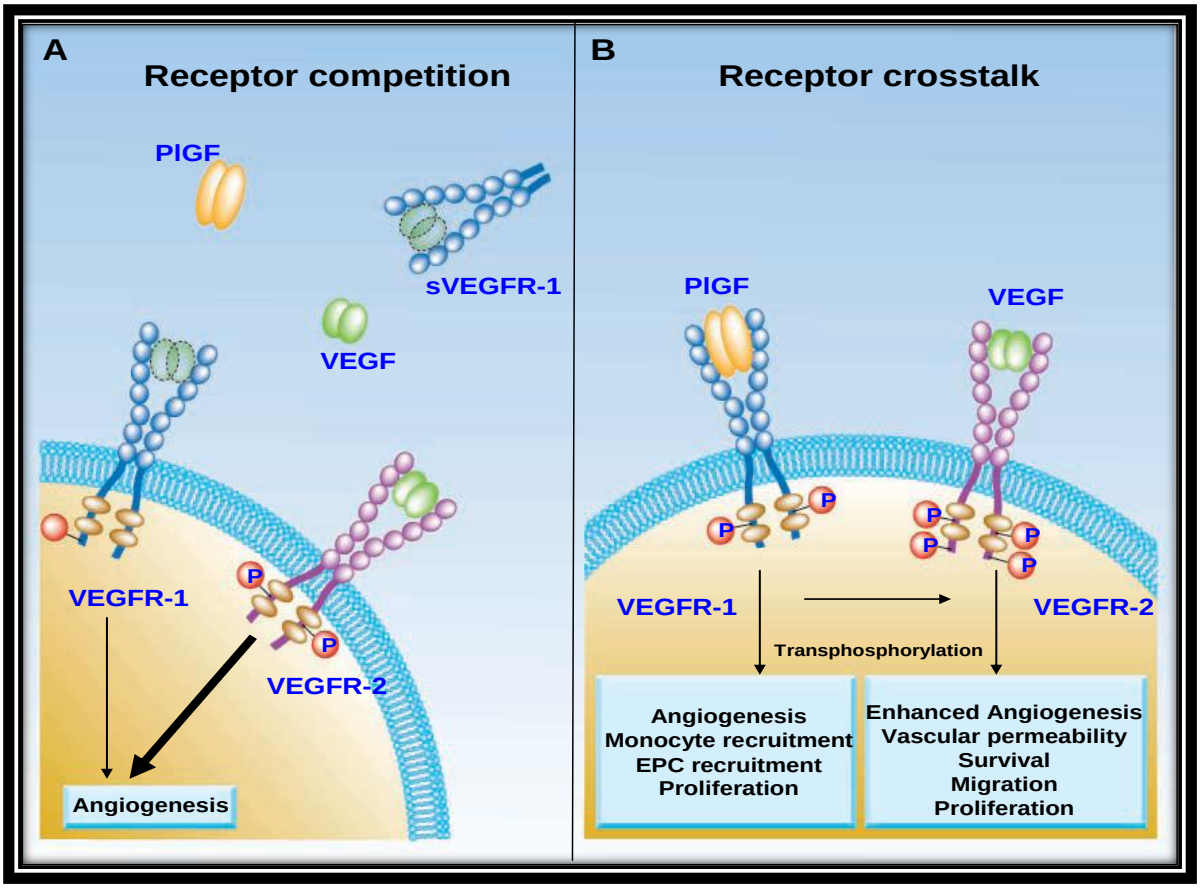


Figure 1.8 Molecular mechanisms of PlGF-induced pro-angiogenic signalling. *[A] VEGFA [VEGF] binds with a higher affinity to [s]VEGFR-1 [designated Flt1in the mouse] than toVEGFR-2 [designated Flk2 in the mouse]. Hence, in physiological conditions, when [B] PlGF expression is minimal, [s] VEGFR-1 acts as a VEGF trap to prevent excessiveVEGFR-2 activation. In cancer, PlGF is up-regulated in cancer cells and stromal cells, which leads to displacement of VEGF from [s] VEGFR-1, increases its bioavailability and binding toVEGFR-2, leading to transmission of a pro-angiogenic signal. B, In addition, PlGF transduces its own signal viaVEGFR-1, evidenced by phosphorylation of distinct tyrosine residues in the intracellular domain ofVEGFR-1.This signal leads to proliferation and migration of tumour cells, ECs, monocytes, and endothelial progenitor cells [EPCs]. Moreover, stimulation of VEGFR-1by PlGF amplifies the VEGF-induced signalling of VEGFR-2 by transphosphorylation of tyrosine residues of VEGFR-2. As a consequence, VEGF-induced angiogenesis, vascular permeability and proliferation, and survival and migration of tumour and ECs are enhanced. (Loges, Schmidt et al. 2009).*

1.8.3 PlGF – pleiotropic activities

PlGF is a pleiotropic factor affecting different cell types; vascular (ECs, pericytes/smooth muscle cells) as well as non-vascular cell types (macrophages, bone marrow-derived progenitors, tumour cells, dendritic cells, fibroblasts, hepatic stellate cells, epithelial cells, neurons, Schwann cells, astrocytes) (Dewerchin and Carmeliet 2012). PlGF is a multitasking cytokine affecting and regulating various biological responses (Figure 1.9). PlGF originally discovered and abundantly expressed in the placenta (Maglione, Guerriero et al. 1991, Hauser and Weich 1993, Cao, Ji et al. 1997). Although trophoblasts are the prime site of synthesis (Khaliq, Li et al. 1996, Vuorela, Hatva et al. 1997) many other cells produce PlGF including ECs, vascular smooth muscle cells, inflammatory cells, neurons, especially when activated (Beck, Acker et al. 2002, Iyer and Acharya 2002, Luttun, Tjwa et al. 2002). PlGF is significantly detected in several organs, including the heart (Iwama, Uemura et al. 2006), lung (Persico, Vincenti et al. 1999), breast (Parr, Watkins et al. 2005), stomach (Chen, Hsieh et al. 2004), prostate (Matsumoto, Suzuki et al. 2003), retina (Feeney, Simpson et al. 2003), thyroid (Viglietto, Maglione et al. 1995) and skin (Failla, Odorisio et al. 2000, Odorisio, Schietroma et al. 2002). PlGF mRNA expression was detected abundantly in trophoblastic cells, which initiate and coordinate vascularisation in the decidua and placenta during early embryogenesis in mice (Achen, Gad et al. 1997). *In situ* hybridization analysis detected PlGF mRNA transcripts extra-embryonically in villous trophoblast, whereas VEGF was expressed in mesenchymal cells within the chorionic plate (Vuorela, Hatva et al. 1997). PlGF-1 facilitates neovascularisation *in vivo* (Ziche, Maglione et al. 1997) and the recruitment of monocytes and ECs (Clauss, Weich et al. 1996, Ziche, Maglione et al. 1997). Unlike VEGF-A, PlGF mRNA expression was not up-regulated by hypoxia in Human Aortic ECs or Human umbilical vein ECs (HUVECs) (Gleadle, Ebert et al. 1995, Cramer, Nagy et al. 2005).

PlGF expression has been reported in various pathological conditions, including cancers: hypervascular renal cell carcinomas, thyroid and germ cell tumours (Takahashi, Sasaki et al. 1994) and in a subset of human meningiomas (Donnini, Machein et al. 1999). Recently, Adini *et al.* found that overexpression of PlGF leads to tumour growth as well as vascular formation. PIGF also induces expression of survival genes and inhibits apoptosis *in vitro*. This study further confirmed that PlGF contributes to tumour angiogenesis by providing functions needed for EC survival (Adini, Kornaga et al. 2002). A recent study documented a cross-talk between tumour cells. Stroma was found to be present in bone metastases of breast cancer and of chronic myeloid leukaemia, in which the tumour cells can educate stroma cells to produce PlGF (Coenegrachts, Maes et al. 2010, Schmidt, Kharabi Masouleh et al. 2011). Although tumour cells respond to PlGF, and some of them even produce PlGF (such as choriocarcinoma cells). Whether tumour cells essentially secrete significant amounts of PlGF is controversial. Conditioned-medium from various cancer cell lines were devoid of PlGF (Figure 4.1).

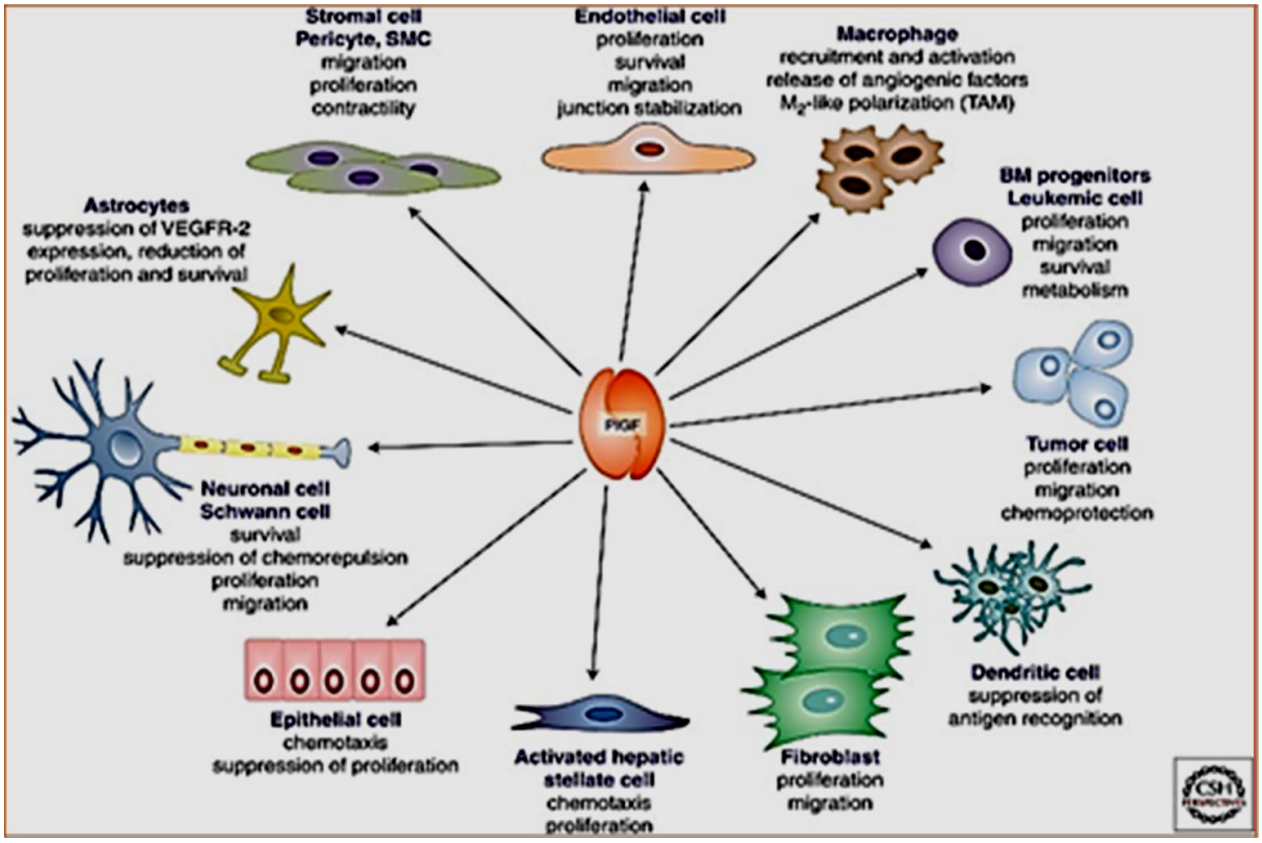


Figure 1.9 Schematic illustration of the pleiotropic actions of PlGF, *including effects on survival, migration, proliferation, metabolism, and activation effects on vascular as well as non-vascular cells. BM, Bone marrow; SMC, smooth muscle cells; TAM, tumour associated macrophage (Dewerchin and Carmeliet 2012).*

1.8.4 Biological Activity and Role of PlGF

PlGF induces various biological activities. PlGF-Knockout (*plgf*$^{-/-}$) mice exhibit normal development and viability with no major vascular defects, indicating that endogenous PlGF is not critical for vascular and embryonic development (Carmeliet, Moons et al. 2001). PlGF contributes to regulating the angiogenic switch, and its significant role in promoting aberrant angiogenesis in a variety of pathologies has gained attention in the past few years (Dewerchin and Carmeliet 2012).

Like VEGF, PlGF exhibits mitogenic activity on cultured ECs (Maglione, Guerriero et al. 1991, Hauser and Weich 1993, Ziche, Maglione et al. 1997) and induces angiogenesis *in vivo*. The angiogenic effects induced by PlGF on ECs are similar to those of the potent classical angiogenic factors, VEGF and FGF-2 (Ziche, Maglione et al. 1997). The role of PlGF in proliferation, migration and permeability of ECs has been controversial; some studies reported PlGF to be ineffective in inducing endothelial cell growth and migration *in vitro* (Park, Chen et al. 1994, Cao, Chen et al. 1996, Kurz, Wilting et al. 1998). However, ECs produce abundant PlGF in culture (Appendix I) (Yonekura, Sakurai et al. 1999). PlGF-deficient mice demonstrated regression of neovascular complexes that had minimal effect on vascular development or normal embryogenesis, yet such a deficiency could reduce collateral vascular growth under pathologic conditions; ischemia, inflammation, and cancer (Carmeliet, Moons et al. 2001). Furthermore, studies demonstrate that loss of PlGF reduced endothelial cell responses (Carmeliet, Moons et al. 2001, Schmidt, Kharabi Masouleh et al. 2011) and ECs from PlGF gene knock-out mice responded to exogenous PlGF (Schmidt, Kharabi Masouleh et al. 2011). The reasons for discrepancies in the reported biological activity of PlGF are unclear, but factors such as sources of PlGF, target cell heterogeneity, or the end points of the assays (Torry, Ahn et al. 1999) could be some of the contributors.

The modulation of PlGF/VEGFR-1 interaction has been reported to affect VEGFR-1 expressing ECs, hematopoietic stem cells, SMCs and inflammatory cells including macrophages (Carmeliet, Moons et al. 2001, Luttun, Tjwa et al. 2002, Autiero, Waltenberger et al. 2003). PlGF stimulates the production of tissue factor and enables chemotaxis in monocytes (Clauss, Weich et al. 1996). PlGF heterodimerisation with VEGF (VEGF: PlGF) was identified in tumour cell supernatants, though less significant than VEGF (Xu, Cochran et al. 2006) (Figure 1.10).

VEGFR-1 raises the possibility that PlGF may act in an autocrine manner to modulate normal trophoblast function (Khaliq, Li et al. 1996). Prominent secretion of PlGF by trophoblasts and the presence of VEGFR-1 receptors on trophoblasts (Shore, Wang et al. 1997) triggered a rapid but transient activation of the stress-activated protein kinase [SAPK], c-Jun-N-terminal kinase [JNK] and p38 kinase pathways. This activation of the signalling pathways resulted in trophoblasts protected from growth factor withdrawal-induced apoptosis (Desai, Holt-Shore et al. 1999). PlGF binding to VEGFR-1 in monocytes led to activation of PI3 kinase/AKT and extracellular signal-regulated kinase (ERK)-1/2 pathways, leading to chemotaxis and the induction of a series of inflammatory cytokines in sickle cell disease (Selvaraj, Giri et al. 2003).

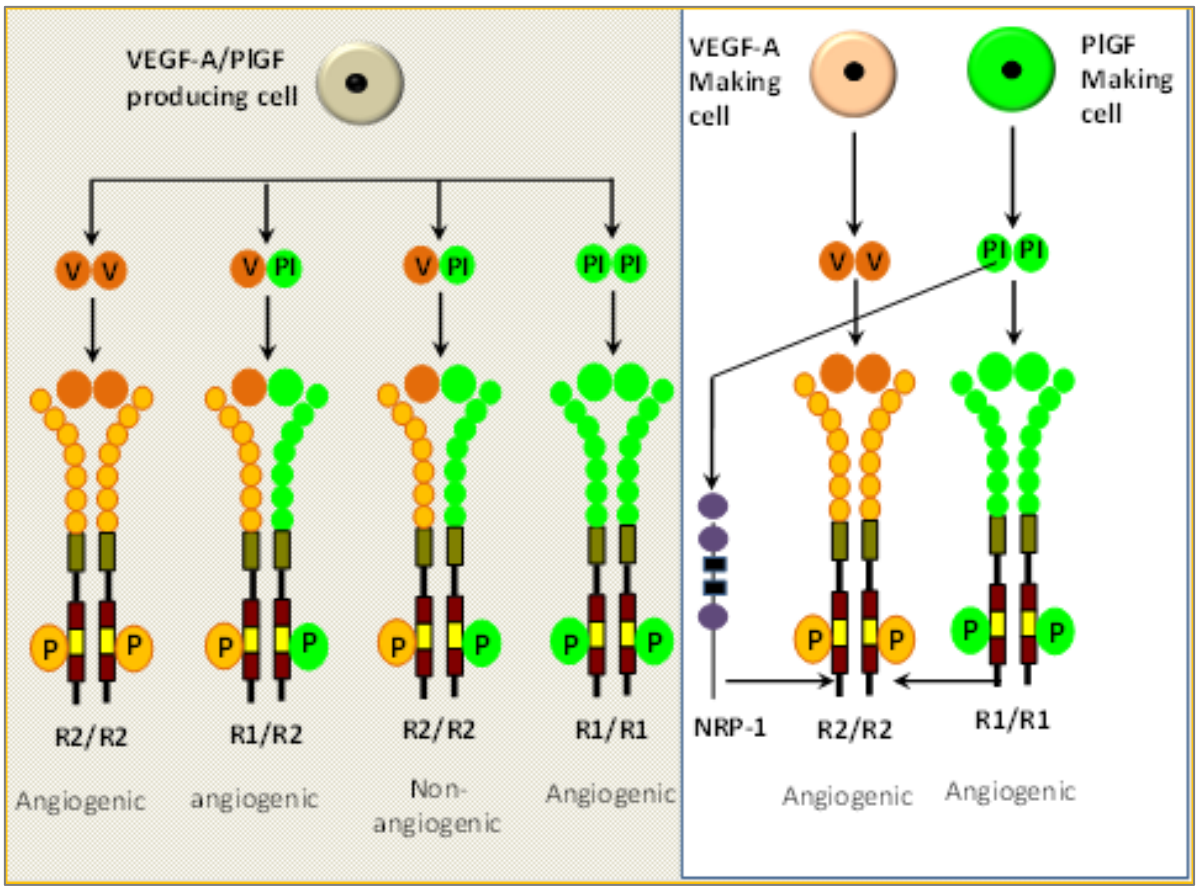


Figure 1.10 Angiogenic response via receptor activation. *Schematic representation of receptor activation elicited by the PlGF:PlGF, VEGF:VEGF homodimers and VEGF:VEGF heterodimer consequent to activation with corresponding receptors. Abbreviations: V=VEGF-A, Pl =PlGF, P= phosphorylation, R1 = VEGFR-1, R2 = VGFR-2, NRP-1 = Neuropilin-1*

Desai and others have reported that PlGF induced ERK-1/2 activation in porcine aortic ECs overexpressing VEGFR-1 (Landgren, Schiller et al. 1998) and in HUVECs (Desai, Holt-Shore et al. 1999). PlGF and VEGF heterodimers exert a stronger mitogenic activity than VEGF homodimers alone in HUVEC (Cai, Ahmad et al. 2003).

PlGF is expressed by migrating keratinocytes and ECs, acting in a paracrine and autocrine fashion on VEGFR-1-expressing endothelium (Failla, Odorisio et al. 2000). Lack of PlGF

resulted in delayed wound closure, indicating that it is required for optimal skin repair (Carmeliet, Moons et al. 2001). Overexpression of this growth factor accelerates wound closure in diabetic mice (Cianfarani, Zambruno et al. 2006).

PlGF via VEGFR-1 alone (Terman, Khandke et al. 1994) promotes monocyte migration (Clauss, Weich et al. 1996), the release of nitric oxide (Bussolati, Dunk et al. 2001), wound healing (Iwama, Uemura et al. 2006) and prolongs survival and stability of capillary-networks (Cai, Ahmad et al. 2003). PlGF also plays a vital role in wound healing and potentiates the VEGF pro-angiogenic function in a synergistic manner (Brown, Yeo et al. 1992, Failla, Odorisio et al. 2000, Carmeliet, Moons et al. 2001). PlGF gene transfer studies into cutaneous wounds of mouse stimulated migration of dermal fibroblasts, enhanced granulation tissue formation, maturation and vascularisation promoting in wound repair (Cianfarani, Zambruno et al. 2006).

Apart from being a chemoattractant for mononuclear cells, PlGF also acts as a potent stimulator of VEGF secretion by monocytes (Bottomley, Webb et al. 2000). PlGF significantly increases mRNA levels of the proinflammatory chemokines, namely, Tumour necrosis factor-alpha [TNF-α], interleukin-1 beta, [IL-1β], Interleukin-8 [IL-8] and monocyte chemoattractant protein-1 [MCP-1] in the peripheral blood mononuclear cells of sickle cell patients when compared to healthy subjects (Perelman, Selvaraj et al. 2003).

1.8.5 Role of PlGF in Pathological angiogenesis

PlGF and its receptor VEGFR-1 have gained increasing attention for its contribution to angiogenesis in pathologies. For example: revascularisation of ischemic tissues by enhancing capillary and collateral vessel formation in the heart, and limb ischemic tissues with PlGF treatment (Luttun, Tjwa et al. 2002, Pipp, Heil et al. 2003, Kolakowski, Berry et al. 2006),

and inhibition of tumour angiogenesis, arthritis and atherosclerosis using anti-VEGFR-1 (Carmeliet, Moons et al. 2001, Luttun, Tjwa et al. 2002).

Analysis of PlGF-deficient mice has revolutionised the understanding of pathological angiogenesis in several disorders, including tumour, ischemic retina and arthritis. This signifies the central role played by PlGF in angiogenesis associated with pathological events (Figure 1.11) (Luttun, Tjwa et al. 2002).

PlGF levels are variably up-regulated in several cancers, including renal-cell carcinoma (Matsumoto, Suzuki et al. 2003), lung cancer (Zhang, Chen et al. 2005), breast cancer (Parr, Watkins et al. 2005), colorectal cancer (Wei, Tsao et al. 2005) and gastric cancers (Chen, Hsieh et al. 2004). This significant role of PlGF in tumour progression has been pursued extensively, in the hope that PlGF might be a useful prognostic marker for cancer progression. Carmeliet and the group have demonstrated that embryonic stem (ES) cell-derived tumours from PlGF deficient mice with C57Bl6 background were small and poorly vascularised (figure 1.9) (Carmeliet, Moons et al. 2001). However, there is no indication that PlGF has a role in tumour formation. PlGF and its receptor VEGFR-1 are the therapeutic targets to treat pathological angiogenesis, pathological arteriogenesis, inflammation, tumour formation and/or vascular leakage. The avb3 integrin, an adhesion receptor for extracellular matrix components with an exposed Arginine-glycine-aspartic acid (RGD) sequence, is an attractive target for anti-angiogenic therapy since it is exclusively present on the cell surface of activated ECs but absent in quiescent endothelium or other cell types (Eliceiri and Cheresh 2000).

Promising results with gene therapy are obtained in therapeutic angiogenesis. Intramuscular gene transfer of $VEGF_{165}$ and intramyocardial administration of an adenoviral vector

expressing the $VEGF_{121}$ cDNA improved collateral vessel development in patients with critical limb ischemia (Arveschoug and Christensen 1999) and coronary artery disease (Rosengart, Lee et al. 1999), respectively. Although VEGF improves myocardial blood flow, it produces endothelium-derived relaxing factor (EDRF)-mediated hypotension (Hariawala, Horowitz et al. 1996), decreases in cardiac output and stroke volume, tachycardia (Yang, Bunting et al. 2000) and, in certain studies, formation of intramural vascular tumours at the implantation site (Lee, Springer et al. 2000). In contrast, both PlGF and FGF-2 has drawn the attention of researchers as a safer and potential therapeutic target for neovascularisation in animal models and trials (Laham, Sellke et al. 1999, Laham, Chronos et al. 2000, Lederman, Mendelsohn et al. 2002, Simons, Annex et al. 2002) (Table 1.3 and 1.4).

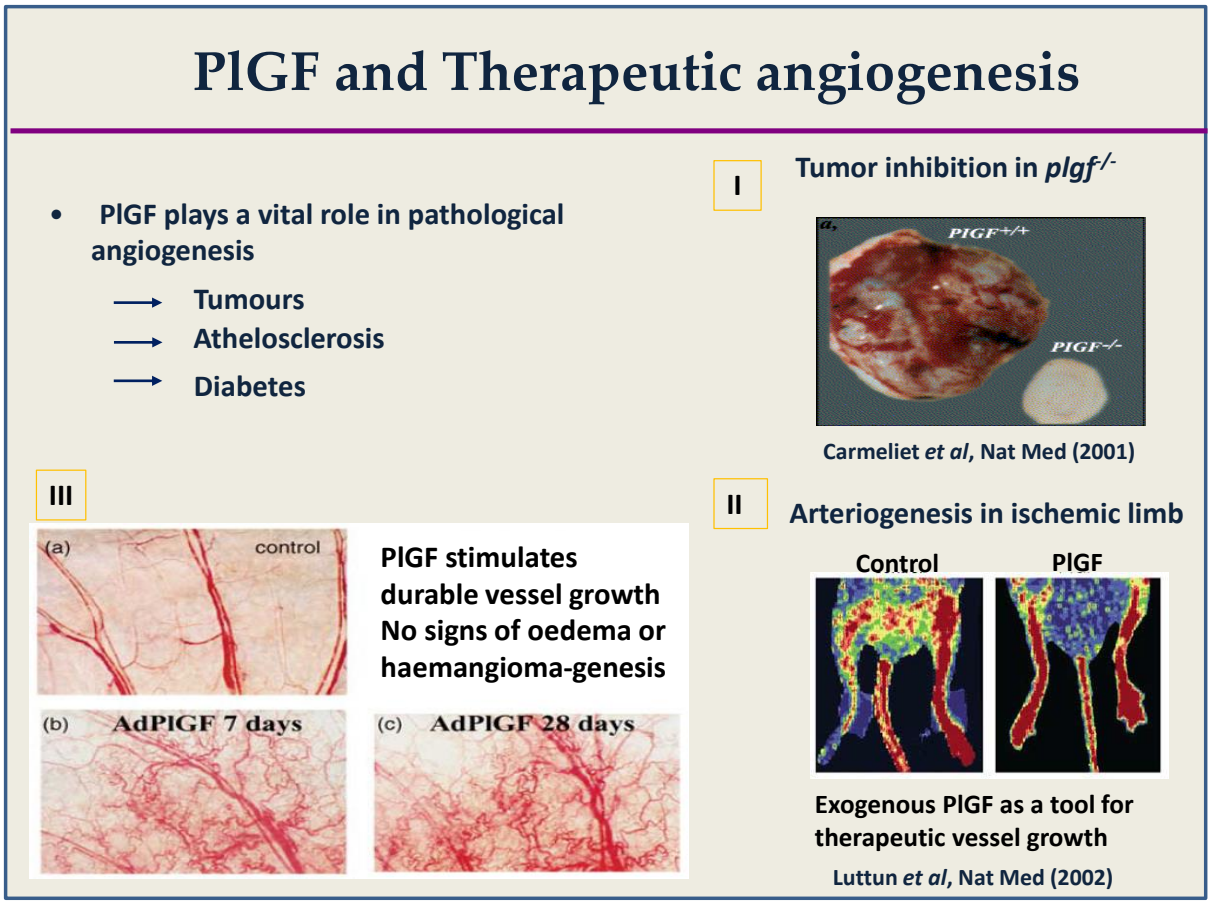


***Figure 1.11 Role of PlGF in therapeutic angiogenesis**. (A) Impaired tumour angiogenesis in Pgf-/-. Macroscopic pictures of wild-type, Pgf–/– [A] ES cell– derived tumours, revealing the haemorrhagic [red] appearance of the large wild-type tumour, in contrast to the small hypovascular [white] Pgf–/– tumours (Carmeliet, Moons et al. 2001). Laser Doppler imaging, revealing that perfusion through the ligated right limb seven days after femoral artery ligation is increased by treatment with hPlGF-2 (Luttun, Tjwa et al. 2002).*

1.8.5.1 Cardio Vascular Disease (CVD) and PlGF

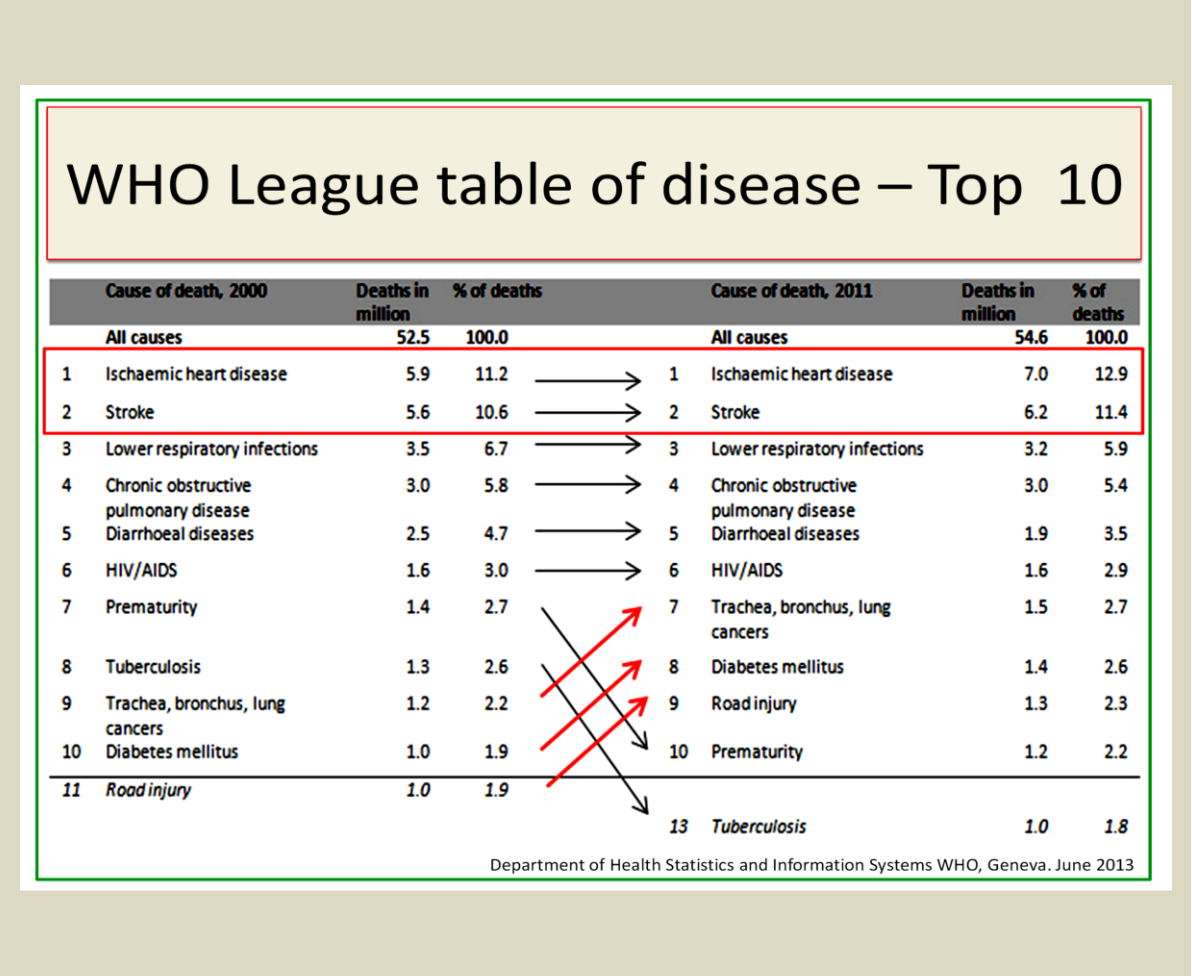
Heart failure remains as one of the leading causes of morbidity and mortality in both Western and developing countries. Atherosclerosis is the disease of hardening and narrowing of the arteries. This progressive disease silently and slowly blocks arteries, putting blood circulation at risk. Atherosclerosis is the usual cause of heart attacks, strokes, and peripheral vascular disease; together they are called cardiovascular disease (CVD). CVD has been projected as the underlying cause for 1 in every 3 deaths in the USA (Roger, Go et al. 2011), and an

estimated 17.3 million CVD-related deaths in 2011, representing 30% of all global deaths (Table 1.2) (WHO 2013). Vascular insufficiency is implicated as the primary cause of a number of CVDs, such as myocardial infarction (MI), peripheral vascular disease (PVD), stroke and ischaemia.

MI results in scar formation with ventricular dilatation and eventually heart failure (Binsalamah, Paul et al. 2011). Rehospitalisation rates due to congestive heart failure as a result of left ventricular dysfunction with MI are still high (Pfeffer and Braunwald 1990). The requirement for these disorders is the return of blood supply to the tissue as fast as possible, to rescue the myocardium in threat of infarct (Martens, Bottelbergs et al. 2012).

PlGF (Carmeliet, Moons et al. 2001, Luttun, Tjwa et al. 2002, Iwama, Uemura et al. 2006, Kolakowski, Berry et al. 2006) is reported to be both pro-angiogenic and pro-arteriogenic without any deleterious side effects, such as hypotension, oedema or hemangio-genesis normally accompanied by systemic VEGF therapy (Oura, Bertoncini et al. 2003, Takeda, Uemura et al. 2009). PlGF-deficient mice display reduced angiogenesis, and inflammation at the border of the infarct after an MI (Carmeliet, Moons et al. 2001).

Correlation studies in patients and mice indicate high expression of PlGF post-acute myocardial infarction (AMI) (Iwama, Uemura et al. 2006). In AMI patients, peripheral plasma PlGF levels on day 3 were significantly higher than in control subjects (Iwama, Uemura et al. 2006) A. The same research group observed PlGF expression was 26.6-fold greater in an AMI model than in sham-operated mice subjected to 40-60 minutes coronary artery ligation (Iwama, Uemura et al. 2006) B. The study reported that the endothelium within the infarcted region is the major site for elevated PlGF expression (Iwama, Uemura et al. 2006).



	Cause of death, 2000	Deaths in million	% of deaths		Cause of death, 2011	Deaths in million	% of deaths
	All causes	**52.5**	**100.0**		**All causes**	**54.6**	**100.0**
1	Ischaemic heart disease	5.9	11.2	1	Ischaemic heart disease	7.0	12.9
2	Stroke	5.6	10.6	2	Stroke	6.2	11.4
3	Lower respiratory infections	3.5	6.7	3	Lower respiratory infections	3.2	5.9
4	Chronic obstructive pulmonary disease	3.0	5.8	4	Chronic obstructive pulmonary disease	3.0	5.4
5	Diarrhoeal diseases	2.5	4.7	5	Diarrhoeal diseases	1.9	3.5
6	HIV/AIDS	1.6	3.0	6	HIV/AIDS	1.6	2.9
7	Prematurity	1.4	2.7	7	Trachea, bronchus, lung cancers	1.5	2.7
8	Tuberculosis	1.3	2.6	8	Diabetes mellitus	1.4	2.6
9	Trachea, bronchus, lung cancers	1.2	2.2	9	Road injury	1.3	2.3
10	Diabetes mellitus	1.0	1.9	10	Prematurity	1.2	2.2
11	*Road injury*	*1.0*	*1.9*				
				13	*Tuberculosis*	*1.0*	*1.8*

Table 1.2 Department of Health Statistics and Information Systems WHO, Geneva. June 2013

Several animal and pre-clinical studies focussed on the role of PlGF in improving cardiac function affected by CVD-related diseases, by promoting wound healing, inducing mobilisation of mononuclear cells and enhancing angiogenesis. A list of some of these studies summarised in Table 1.3.

Conversely, studies reported correlations between adverse cardiac outcomes in long-term follow-up patients with acute coronary syndrome (ACS) and plasma PlGF levels (Dewerchin and Carmeliet 2012). PlGF is a recruiter of macrophages and monocytes, a role that may result in on-going inflammatory processes in the coronary artery wall. This on-going process

may trigger adverse cardiovascular events (Heeschen, Dimmeler et al. 2004, Lenderink, Heeschen et al. 2006, Dewerchin and Carmeliet 2012).

Species	Patients/ Animal Model	PlGF detection/ Therapy	Outcome	Author
Human (55)	AMI	↑ PlGF protein Infarct region	+ correlation to LVEF changes	Iwama, H *et al* 2006
Mouse	IR - 40 -60 mins coronary artery ligation MI (permanent ligation)	↑ PlGF mRNA	Recruitment and Activated macrophages Wound healing	Iwama, H *et al* 2006
Mouse	MI - Ligation of left anterior descending (LAD) coronary artery	1×10^9 pfu/ml AdPlGF Systemic administration	↑capillary density in the infarct border vessel enlargement in the remote myocardium promoted adaptive remodelling	Roncal, C *et al* 2008
Human (98)	Ischaemic cardiomyopathy	↑ Plasma PlGF (in accordance to severity of heart failure)	Myocardial hypoxia and/or intravascular inflammation = ↑PlGF , Stimulate angiogenesis	Nakamura, T *et al* 2009
Mouse (95)	MI (coronary artery ligation)	rhPlGF (10µg) subcutaneously Osmotic pump 3 days	↓ Infarct area ↑ CD31 +ve cells Angiogenesis α Smooth muscle actin +ve vessels Arteriogenesis Survival	Takeda, Y *et al* 2009

Table 1.3 Role of PlGF in improving cardio-protective function in CVD

1.8.5.2 PlGF and Preeclampsia (PE)

PE is a systemic condition of the maternal endothelium, with the clinical presentation of hypertension and proteinuria (Friedman, Taylor et al. 1991) that affects 3-7 % (Enkhmaa, Wall et al. 2016) of all pregnancies. PE remains one of the leading causes of maternal-foetal mortality and morbidity (Schaap, van der Wal et al. 1993, Brewster, Orsi et al. 2008) even in developed countries. Numerous studies have reported an imbalance of angiogenic and anti-angiogenic proteins to be a key influence leading to the maternal symptoms of PE (Ivan, Kondo et al. 2001, Maynard, Min et al. 2003, Ahmad and Ahmed 2004, Levine, Maynard et al. 2004, Levine, Lam et al. 2006, Chaiworapongsa, Romero et al. 2008). Endothelial

dysfunction is responsible for the clinical symptoms of the disorder (Ueda, Hirai et al. 1996) include poor placental vascular remodelling and subsequent placental hypoxia (Schaap, van der Wal et al. 1993), oxidative stress (Roberts and Redman 1993, Schaafhausen, Yang et al. 2013) and excessive inflammation (Redman, Sacks et al. 1999).

Circulating levels of the anti-angiogenic protein sVEGFR-1, with antagonist activity to both VEGF and PlGF, is reported to be increased in the placenta (Maynard, Min et al. 2003, Tsatsaris, Goffin et al. 2003) and serum (Zhou, McMaster et al. 2002, Koga, Osuga et al. 2003) of women with PE, and is associated with severity of the disease. sVEGFR-1 was detected long before the onset of the clinical manifestations of PE (Hunter, Aitkenhead et al. 2000, Maynard, Min et al. 2003, Tsatsaris, Goffin et al. 2003, Levine, Lam et al. 2006). In fact, evidence of low PlGF levels in the first trimester, before an increase in sVEGFR-1 levels, is stated as a predominant risk factor pertaining to this disorder (Levine, Maynard et al. 2004, Thadhani, Mutter et al. 2004).

Maternal circulation levels of VEGF have been reported by different investigators with conflicting results including increase, decrease or no change in the levels of VEGF in women with healthy pregnancies compared to pregnancies complicated by PE (Baker, Krasnow et al. 1995, Sharkey, Cooper et al. 1996, Kupferminc, Daniel et al. 1997, Lyall, Greer et al. 1997). In both PE and acute pyelonephritis (AP), the most common septic shock during pregnancy, circulating levels of PlGF are reported to be dramatically reduced (Polliotti, Fry et al. 2003, Chaiworapongsa, Romero et al. 2009) and have been associated with inflammation.

Excessive inflammatory cell activation, beyond the robust inflammatory response, observed during normal pregnancies, has been proposed as a driving force that contributes to the endothelial dysfunction that was seen in PE (Freeman, McManus et al. 2004). This was

further supported by expression array studies, indicating widespread up-regulation in the expression of cytokines and their receptors in the placentas of PE complicated pregnancies (Pang and Xing 2003). Abnormal production of cytokines and an asymmetry between Th1 type cells that produce pro-inflammatory cytokines, such as, TNF-α, IL-1β, IL-12 to that of Th2 type cells cytokines that produce IL-4, IL-6, IL-10 yield to vascular damage and PE (Conrad, Miles et al. 1998, Ellis, Wennerholm et al. 2001, Freeman, McManus et al. 2004). Increased circulating levels of the pro-inflammatory cytokine TNF-α, and its soluble TNF receptor 1 [sTNF-R1], are detected in PE as well as AP (Chaiworapongsa, Romero et al. 2009) and correlate with the severity of the disease (Schipper, Bolte et al. 2005). TNF-α induced the release of sVEGFR-1 from cultured placental explants (Ahmad and Ahmed 2004).

A very recent study demonstrated that continuous administration of TNF-α for two weeks at mid-gestation to a cytokine-induced model of pregnant baboons caused changes that are seen in human PE, i.e. elevation in blood pressure and proteinuria (Ueda, Hirai et al. 1996). The treated pregnant animals also developed elevated plasma, and placental mRNA expression of sVEGFR-1 and soluble endoglin (sEng), a soluble form of transforming growth factor receptor TGF-β, compared to pregnant saline controls or in non-pregnant TNF-α treated animals. These results identify a link between cytokines, placental dysfunction and endothelial dysfunction resulting in the onset of PE-like symptoms. Furthermore, Sunderland and group postulate that hypertension and proteinuria in the setting of increased sVEGFR-1 can be caused by an increase in TNF-α alone (Sunderland, Thomson et al. 2011).

Although extensive animal studies and epidemiological studies have been performed suggesting multiple factors leading to PE, to our knowledge studies assessing the impact of

inflammatory cytokines on angiogenic factors, such as the effect of TNF-α on PlGF, and key factors in PE are none.

1.8.5.3 Role of PlGF in inflammatory disorders

Inflammation is increasingly recognised as a central component of the pathophysiology of several diseases. Inflammation manifested with the elevated levels of inflammatory cytokines, activation of neutrophils, monocytes and ECs (Pober and Sessa 2007). Inflammation is a complex stereotypical set of reactions expressed in response to the damage of the cells and vascularised tissues. Angiogenic mediators, including growth factors, cytokines, matrix components and other contributors, such as hypoxia, have been implicated in capillary formation. Additionally, circulating levels of angiostatic activating agents, namely anti-inflammatory cytokines and other growth factors, contribute towards sustenance of controlled angiogenesis (Auerbach and Auerbach 1994, Szekanecz and Koch 2001, Szekanecz and Koch 2007). Perturbing this equilibrium between angiogenesis and inflammation may lead to inflammatory diseases, such as PE (Sharma, Satyam et al. 2007), rheumatoid arthritis (Folkman 1995), psoriasis (Braverman and Sibley 1982) or Crohn's disease (Kanazawa, Tsunoda et al. 2001). The process of inflammation in association with angiogenesis is essential for tissue repair (Risau 1994, Carmeliet and Jain 2000, Streit, Velasco et al. 2000). Several pro-angiogenic factors that contribute to the inflammatory vascular response including PlGF (Clauss, Weich et al. 1996, Luttun, Autiero et al. 2004), VEGF (Detmar, Brown et al. 1994), FGF (Kanazawa, Tsunoda et al. 2001), and angiopoietins (Kuroda, Sapadin et al. 2001) have been reported.

In rheumatoid arthritis (RA) the inflamed synovium has detectable levels of growth factors, inflammatory cytokines, chemokines, adhesion molecules as well as matrix-degrading

proteases (Bodolay, Koch et al. 2002). PlGF and VEFGR-1 were detected on inflammatory mediators and ECs in the inflamed synovium in animal models mimicking human arthritis (Courtenay, Dallman et al. 1980). Excessive or insufficient angiogenesis enhances a number of inflammatory processes, and the two are often co-dependent (Jackson, Seed et al. 1997). The activated endothelium is an integral part of the inflammatory response, and is due to the apparent co-dependency of inflammation and angiogenesis; compounds that block the angiogenic response may also prove beneficial for inflammatory diseases.

1.9 Fibroblast Growth Factors [FGF]

Like PlGF, FGFs are pleiotropic factors belonging to the RTK class IV of angiogenic factors and were among the first molecules discovered to exert proliferation, migration and differentiation of vascular cells, both *in vitro* as well as *in vivo* (Bikfalvi, Klein et al. 1997). FGFs manifest their biological effects on different cell types, including ECs. The FGF family consists of 23 members (Nishimura, Utsunomiya et al. 1999) that share around 55% homology of the amino acid residues, ranging between 18- to 30-kDa proteins with a high affinity for HS domains (Abraham, Whang et al. 1986, Liekens, De Clercq et al. 2001). HS is critical for the biological effect for some of the members of FGF (Spivak-Kroizman, Lemmon et al. 1994, Galzie, Kinsella et al. 1997). FGF-FGFR interactions and signalling require heparin sulphate proteoglycans [HSPGs] as co-receptors (Powers, McLeskey et al. 2000) and demonstrate developmental, tissue and cell-specific regulation. The prototypes; FGF-1 and FGF-2, are unique due to the lack of a conventional leader sequence. Consequently, secretion by the classical secretory pathway via endoplasmic reticulum does not occur (Basilico and Moscatelli 1992, Friesel and Maciag 1995).

1.9.1 FGF-2 [Basic FGF]

FGF-2 is a potent angiogenic factor modulating cell proliferation and/or differentiation and survival in a variety of cell types, including those with stem cell properties, thus affecting various organ systems and biological processes (Kardami, Detillieux et al. 2007).

1.9.1.1 Structure and Function

The human FGF-2 gene is located on chromosome 4 (Mergia, Eddy et al. 1986), and consists of three exons separated by two introns. FGF-2 is a cationic mitogen weighing about 18,000 kDa, measuring 155 amino acid [aa] residues in length (Abraham, Whang et al. 1986). FGF-2 was one of the first angiogenic growth factors characterised and studied extensively (Presta, Moscatelli et al. 1986). FGF-2 has four alternate CUG [leucine] start codons that provide N-terminal extensions of 41, 46, 55 or 133 aa, resulting in proteins of 22 kDa [196 aa], 22.5 kDa [201 aa], 24 kDa [210 aa] and 34 kDa [288 aa], respectively (Arnaud, Touriol et al. 1999).

Human FGF-2 gene encodes multiple FGF-2 isoforms, ranging from 18, 000 to 24, 000 kDa (Florkiewicz and Sommer 1989). Both low and high molecular weight subtypes demonstrate angiogenic activity *in vivo*, inducing cell proliferation, chemotaxis and production of urokinase plasminogen activator in cultured ECs (Presta, Moscatelli et al. 1986, Eliceiri, Puente et al.). In general, the 155 aa/18 kDa or the low molecular weight [LMW] form is considered cytoplasmic [and secretable] (Bugler, Amalric et al. 1991, Florkiewicz, Majack et al. 1995, Delrieu 2000), whereas the high molecular weight [HMW] forms are considered nuclear in the target.

1.9.1.2 Receptor Binding

To exert their biological activity, FGF's interaction with tyrosine kinase FGF receptors [FGFR] is essential. FGFRs contain two or three immunoglobulin-like domains and a heparin-binding sequence (Johnson, Lee et al. 1990). FGF-2 binds specifically to the distinct splice variant FGFR-1 isoform of the different FGFRs (Zhang, Ibrahimi et al. 2006). *In vitro* ECs from different origins express FGFR-1, whereas FGFR-2 is less frequently expressed (Presta, Dell'Era et al. 2005).

1.9.1.3 Expression and Regulation

FGF-2 secreted as a monomer and is either sequestered on matrix glycosaminoglycans or cell surface HS. The dimerization of the monomeric FGF-2 occurs in a non-covalent side-to-side configuration by the cell surface HS. This dimerised FGF-2 can activate FGF receptors (Herr, Ornitz et al. 1997, Okada-Ban, Thiery et al. 2000, Ornitz 2000).

FGF-2 is expressed in several cell types, including platelets, visceral and vascular smooth muscle cells (SMC), epithelium lining of the colon, cardiac muscle cells, bronchus epithelium, megakaryocytes, neurones, and cerebellar Purkinje cells (Florkiewicz, Shibata et al. 1991). In addition, FGF-2 is also expressed in ECs (Cordon-Cardo, Vlodavsky et al. 1990, Yu, Biro et al. 1993), fibroblasts [plus extracellular matrix], numerous embryonic mesodermal and neuroectodermal tissues (Cordon-Cardo, Vlodavsky et al. 1990), glomerular parietal epithelial cells and podocytes, mast cells, astrocytes, $CD4^+$ and CD8 T cells (Cordon-Cardo, Vlodavsky et al. 1990).

1.9.2 Biological Effects of FGF-2 in Pathologies

FGF-2 has been reported to be found in extracellular matrices and the extracellular circulation (Hanahan and Folkman 1996, Fannon, Forsten et al. 2000). Under normal conditions, the

levels of FGF-2 found are relatively low (Fannon, Forsten et al. 2000). However, these levels are substantially elevated in cases of disease and cancer (Riley, Savage et al. 1993, Fannon, Forsten-Williams et al. 2003). For example, high levels of FGF-2 have been found in the circulation of ECs in pathologies, such as Kaposi's sarcoma and hemangiomas, implicating the autocrine role of FGF-2 (Presta, Dell'Era et al. 2005). FGF-2 has also been associated with tumour angiogenesis and a wide spectrum of cancers (Chodak, Hospelhorn et al. 1988, Nguyen, Watanabe et al. 1994, Meyer, Yu et al. 1995, Toi, Taniguchi et al. 1996). The temporal and spatial distribution of FGF-2 is fundamental, not only for normal development but for disease control as well (Fannon, Forsten et al. 2000, Lalani, Wong et al. 2005, Lee, Silva et al. 2011), that can be regulated by HSPGs. Isolated ECs from FGF-2 null mice exhibited defects in cell migration (Pintucci, Moscatelli et al. 2002), resulting in delays in wound healing (Ortega, Ittmann et al. 1998).

FGF-2 is one of the most widely used pro-angiogenic growth factors for the restoration of cardiac function (Kardami, Detillieux et al. 2007, Liao, Porter et al. 2007). Cardiomyocytes express functional FGF-2 receptors and, upon activation by FGF-2, trigger multi-signalling cascades resulting in cytoprotection, DNA synthesis, and negative inotropic effect (Ishibashi, Urabe et al. 1997, Padua, Merle et al. 1998). Several pre-clinical and animal studies were carried out to investigate the cardio-protective role of FGF-2, which are summarised in Table 1.3. FGF-2-mediated angiogenesis is dependent on VEGFR-1 [Flt-1] activity (Kanda, Miyata et al. 2004). Kanda and colleagues demonstrated that blocking VEGFR-1 inhibited FGF-2-mediated tube formation in cultured HUVEC (Kanda, Miyata et al. 2004). The biological implications of FGF-2 are vast and play a significant role in vascular development as well as an exogenous therapeutic tool. Angiogenesis is a complex process that is regulated by several pro- and anti-angiogenic factors. FGF-2 and PlGF share Janus-like roles in pathological and

therapeutic angiogenesis, such as inducing wound healing, leukocyte recruitment, collateral growth post-MI. They have been identified as contributors to the progression of atherosclerosis (Figure 1.12) (Luttun, Tjwa et al. 2002, Khurana, Moons et al. 2005, Virag, Rolle et al. 2007, Liu, Tang et al. 2013).

Species	Patients/ Animal Model	FGF-2	Outcome	Author
Rats (86)	MI (permanent surgical coronary occlusion)	FGF-2 : 0.2 and 2 µg injected (in a total volume of 100 µl)	protection from acute and chronic cardiac dysfunction and damage PKC mediated	Kardami *et al* 2001
Mouse (95)	MI (coronary artery ligation)	FGF2- KO	↑ Infarct wall thinning Ventricle dilation, hypertrophy fibroblast proliferation, fibrosis ↓ EC proliferation, Vascular density,	Virag *et al* 2007
		FGF2-Tg →	Reversed + Preserved LV function	
Pigs (40)	MI (Balloon inflation at left ascending coronary artery for 90 min)	FGF-2 controlled release using hydrogels	formation of microvascular network Myocardial perfusion Improved LV function Reduced infarct size	Takehara *et al* 2008
Human (38) FGF-2 (16) IR (22) - 38	1 graftable obstructed CA & 1 ischemic, viable myocardial territory supplied by a major coronary branch, which was not amenable to complete revascularization	CABG+500 µg of human rFGF-2 Administered intramyocardially at 15 sites of targeted myocardial territory 27-gauge needle	Myocardial neovascularizaton and collateral growth Myocardial perfusion in myocardial territories injected with FGF-2 Coronary angiographic and myocardial scintigraphic analysis	Katayama *et al* 2010
Rats (40)	MI (permanent ligation of left coronary Artery)	FGF-2 mRNA	FGF-2 mRNA increased in infarct zone up to 14 days. No change in FGF-2 levels in non-infarcted zone FGF-2 increased at day 7 post MI Expressed primarily in endothelial cells in the newly formed vessels at the border zone and infarcted myocardium	Zhao T *et al* 2011

Table 1.4 Role of FGF-2 in improving cardio-protective function in CVD

The cellular activities in response to the paracrine and autocrine interactions of these angiogenic growth factors remain unclear. However, all of these findings suggest that a subtle balance exists between the beneficial effects of angiogenic therapy (including revascularisation, cardiac protection and wound healing), versus detrimental effects requiring

anti-angiogenic therapy (including inflammatory angiogenesis leading to rheumatoid arthritis, atherosclerosis or tumour angiogenesis) exerted by PlGF, FGF-2 and their respective receptors.

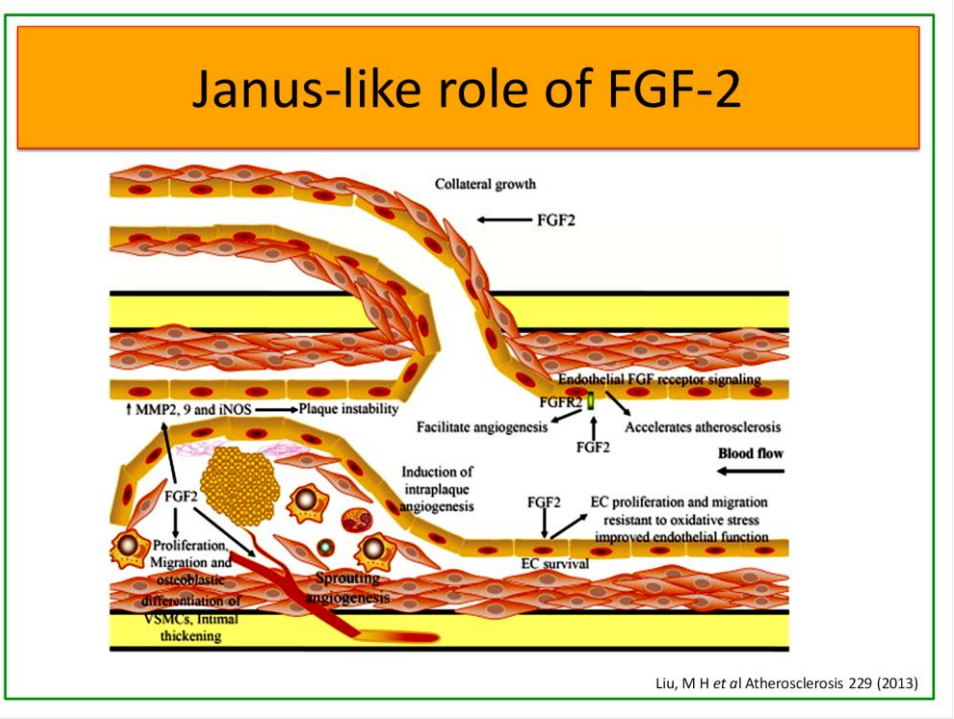


Figure 1.12 Janus-like role of FGF-2 in the atherosclerotic lesions of coronary artery disease. *In a normal vessel wall, the circulating or autocrine FGF-2 binds to its receptor on the surface of ECs (ECs). This process involved in the resistance of ECs to oxidative stress improves endothelial function and promotes EC survival. When expressed at high concentrations because of protein delivery, FGF-2 induces collateral growth, which helps restore blood circulation to the infarcted areas. In atherosclerotic lesions, FGF-2 is released by activated inflammatory cells, ECs and vascular smooth muscle cells (VSMCs). The role of these cells in advanced lesion development is undetermined. FGF-2 may participate in the stimulation of early stage angiogenesis of microvessels entering from the adventitia. FGF2 may induce the proliferation, migration and osteoblastic differentiation of VSMCs. FGF2 may also cause intimal thickening and promote plaque vulnerability. Endothelial FGF receptor (FGFR) signalling has a dual role in the cardiovascular system (Liu, Tang et al. 2013).*

1.10 Signal transduction of pro-angiogenic growth factors

Therapies to maintain the balance between the angiogenic stimulators and inhibitors have been a fundamental goal of several research groups and pharmaceutical industries to try to

help alleviate disease. One of the major ways to achieve this is by manipulating the angiogenic growth factor ligands and/or receptors that elicit signal transduction pathways, in order to inhibit or propagate induction of angiogenesis or arteriogenesis in pathologies. The discovery of the downstream signal transduction pathways for RTKs has led to the development of many newly targeted agents. The three major signal transduction pathways for angiogenic growth factors are the Ras/Raf/MAPK pathway, PI3K/Akt/mTOR pathway, and PKC pathway (Figure 1.13). These multiple signalling pathways can work independently or in concert to elucidate the signal transduction, resulting in cellular activities. Several studies focussed on manipulating angiogenesis by regulating the signalling pathways that are unique to the growth factor ligands or their respective receptors.

In homoeostasis, a fine balance exists between pro- and anti- angiogenic factors that tightly regulate angiogenesis (Bergers and Benjamin 2003). Tumour angiogenesis or pathological angiogenesis results from an uncontrolled excess of pro-angiogenic factors (e.g. hypoxia and chemo-gradient of growth factors) that stimulate angiogenesis. Common signalling targets that regulate cell proliferation and subsequent angiogenesis are crucial for metastasis and their identification could, therefore, aid in controlling the disease progression

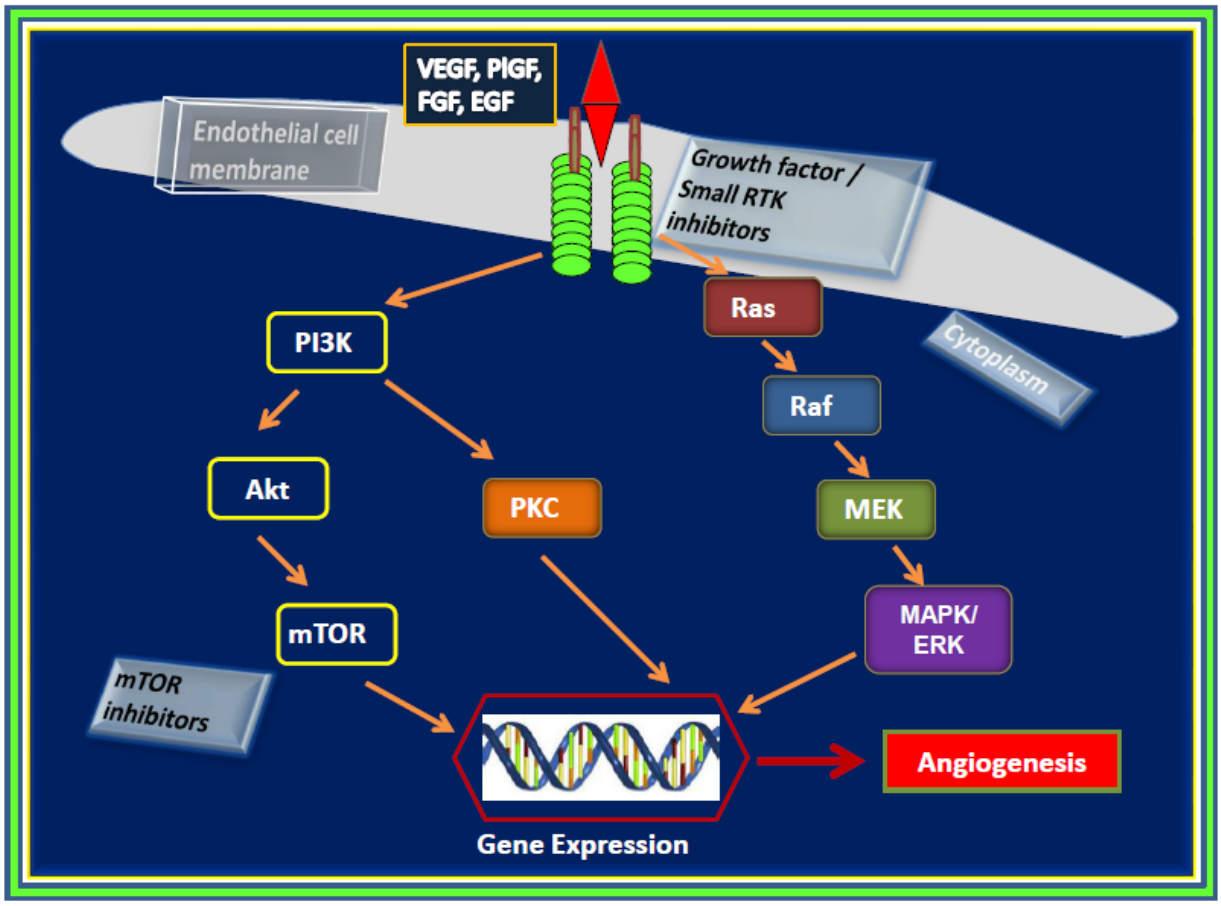


Figure 1.13 Major signal transduction pathways for angiogenic growth factors *RTK include the Ras/Raf/MAPK pathway, PI3K/Akt/mTOR pathway and PKC pathway. The receptors and their signal transduction pathways are potential therapeutic targets for anti-angiogenesis therapy. (Adapted from US Pharma. 2010; 35(7) (Oncology Suppl): 4-9).*

1.11 Inflammatory Angiogenesis

There is a growing body of experimental evidence linking systemic inflammation and endothelial dysfunction (Table 1.5; Figures 6.1 and 6.13). When exposed to certain pathogenic pro-inflammatory stimuli, the endothelium expresses leukocyte chemotactic factors, adhesion molecules and inflammatory cytokines. Inflammation is a complex set of reactions expressed in response to damage to the cells and vascularised tissues. During inflammation, angiogenesis is initiated by the activation of different cell populations, which release a variety of angiogenic factors that exhibit both pro-inflammatory and pro-angiogenic effects (DN 2010).

The process of inflammation in association with angiogenesis is essential for tissue repair (Risau 1994, Carmeliet and Jain 2000, Streit, Velasco et al. 2000). Several pro-angiogenic factors that contribute to the vascular inflammatory response have been reported, including PlGF (Clauss, Weich et al. 1996, Luttun, Autiero et al. 2004), VEGF (Detmar, Brown et al. 1994), FGF (Kanazawa, Tsunoda et al. 2001), and angiopoietins (Kuroda, Sapadin et al. 2001). ECs are an active part of the inflammatory response and are known as inflammatory amplifiers. Adhesion molecules on the EC surface are exposed after receiving stimulatory signals from pro-inflammatory cytokines (Mai, Virtue et al. 2013). In contrast to the host defence manifested protective inflammation, chronic inflammation causes substantial tissue damage which triggers pathological angiogenesis; chronic inflammation promotes a continuous recruitment of inflammatory cells, thereby exacerbating inflammation and damage (Costa, Incio et al. 2007). On the other hand, angiogenesis itself can up regulate inflammation. It is well documented that the pro-angiogenic and anti-angiogenic factors represent the balance between the resting and activated vascular endothelium. Systemic inflammation and endothelial dysfunction related human studies listed in Table 1.5. Disturbing this equilibrium

may lead to chronic inflammatory diseases, such as preeclampsia (PE) (Sharma, Satyam et al. 2007), RA (Folkman 1995), psoriasis (Braverman and Sibley 1982) or Crohn's disease (Kanazawa, Tsunoda et al. 2001). Excessive angiogenesis or inhibition of angiogenesis enhances a number of inflammatory processes, and the two are often co-dependent (Jackson, Seed et al. 1997). Because of the apparent co-dependency of inflammation and angiogenesis, compounds that block the angiogenic response may also prove beneficial for inflammatory diseases. VEGF-R1, and its ligands VEGF and PlGF participate in an important role in monocyte chemotaxis and promote recruitment of circulating endothelial precursor cells from bone marrow (Hattori, Heissig et al. 2002, Holmes and Zachary 2004). PlGF can also contribute to inflammation by acting as a survival factor for monocytes and macrophages (Adini, Kornaga et al. 2002).

A large number of diseases, such as RA, heart disease, tumour progression and metastasis, PE, obesity and trauma that is associated and not associated with any external stimuli or injury, are known to induce pro-inflammatory cytokines. Amplified tissue infiltration by macrophages is a dramatic and common feature of acute/chronic inflammation and angiogenesis.

ECs express a broad spectrum of cytokines and chemokines, including TNF-α, IL-1β, IL-3, IL-5, IL-6, IL-8, IL-11, IL-15 (Krishnaswamy, Kelley et al. 1999). These are all pro-inflammatory mediators in potentiating an inflammatory response, including inflammatory angiogenesis by inducing cytokine and growth factor secretion by ECs and non-ECs to the site of inflammation (Krishnaswamy, Kelley et al. 1999). Several cytokines and growth factors that are part of the inflammatory angiogenesis have pro-thrombotic and fibrogenic effects (Krishnaswamy, Kelley et al. 1999).

IL-1β and TNF-α, in particular, are effective in triggering the expression of pro-inflammatory genes in various types of cells (Kumar 2012).

Stimuli for inflammatory response	Measurement/Assessment	Response	Comment	Ref
Salmonella typhi vaccination	Vascular Function	-Acute inflammatory response - Increased levels of inflammatory response	Short-term impairment of endothelium-dependent dilation in conduit and resistance vessels that paralleled the inflammatory response	(Hingorani, Cross et al. 2000)
Salmonella typhi capsular polysaccharide vaccine 0.025 mg	Effect of Asprin on endothelial dysfunction	-TNF-α increased, peaking at 4 hours after vaccination. -Aspirin prevents inflammation-induced endothelial dysfunction by modulation of the cytokine cascade	Effect of oral aspirin (1.2 g) pre-treatment on vaccine-induced endothelial dysfunction. -Effect of local intra-Arterial aspirin on established vaccine-induced endothelial dysfunction	(Kharbanda, Walton et al. 2002)
1 hour exposure to TNF-α, IL-1β, or endotoxin	Hand veins	-Impaired endothelial function in hand veins of healthy subjects	Endothelial dysfunction persisted for 48h and up to 7 days to recover	(Bhagat, Moss et al. 1996)

Table 1.5 Human studies of systemic inflammation and endothelial dysfunction

Additionally, both IL-1β and TNF-α exhibit pro-coagulant activity by inducing endothelial expression of tissue factor, and they share overlapping functions in the acute as well as the chronic inflammatory response (Voronov, Carmi et al. 2014). TNF-α is probably the most studied candidate for initiating inflammatory angiogenesis. In physiological conditions, TNF-α is hardly detected. However, in pathological disorders, TNF-α concentration amplifies to abnormally high levels (Lee, Zaske et al. 2011) (Table 1.6). Such high levels are toxic and could lead to systemic vascular dysfunction if untreated (Lee, Zaske et al. 2011).

Pathologies	Pathology Plasma level of TNF-α [pg/mL]
Physiological conditions	1.0
Rheumatoid arthritis	1000
Atherosclerosis	1000–2000
Colorectal cancer	2
Pancreatic adenocarcinoma	30
Non-small cell lung cancer	10
Chronic lymphocytic leukemia	20
Prostate cancer	4
Metastatic prostate cancer	6
Breast cancer	5
Pre-eclampsia	200
Obesity	10
Hemorrhagic shock alone	60
Hemorrhagic shock with subsequent MOF	160

Table 1.6 List of common pathologies presenting higher plasma levels of circulating TNF-α and corresponding concentrations. (Adapted from Sei-Lee, Internation Journal of nanomedicine, 2012).

Chapter 2 Aims of the study

❑ Aims

Chapter 4

- ❑ Identify growth factors and cytokines regulating endothelial PlGF levels

Chapter 5

- ❑ Delineate the underlying signalling mechanism governing FGF-2-mediated endothelial PlGF release

Chapter 6

- ❑ Analyse the effect of FGF-2 on endothelial PlGF from micro- and macro-vascular endothelial cells
- ❑ Examine the role of PlGF in FGF-2-mediated angiogenesis *in vitro* and *ex vivo* mouse PlGF gene knock out models

Chapter 7

- ❑ Study the impact of pro-inflammatory cytokines on endothelial PlGF release in micro and macro-vascular endothelial cells.
- ❑ Examine the role of endogenous PlGF on endothelial cell survival

Chapter 3. Materials and Methods

3.1 Materials and Methods

Equipment used for this study and its suppliers are documented in Appendix III. Protocols for production of all solutions and buffers are detailed in Appendix IV.

Experimental methods

Various experimental methods used in this study are listed in Figure 3.1. Detailed description and application of each method used can be found in this chapter.

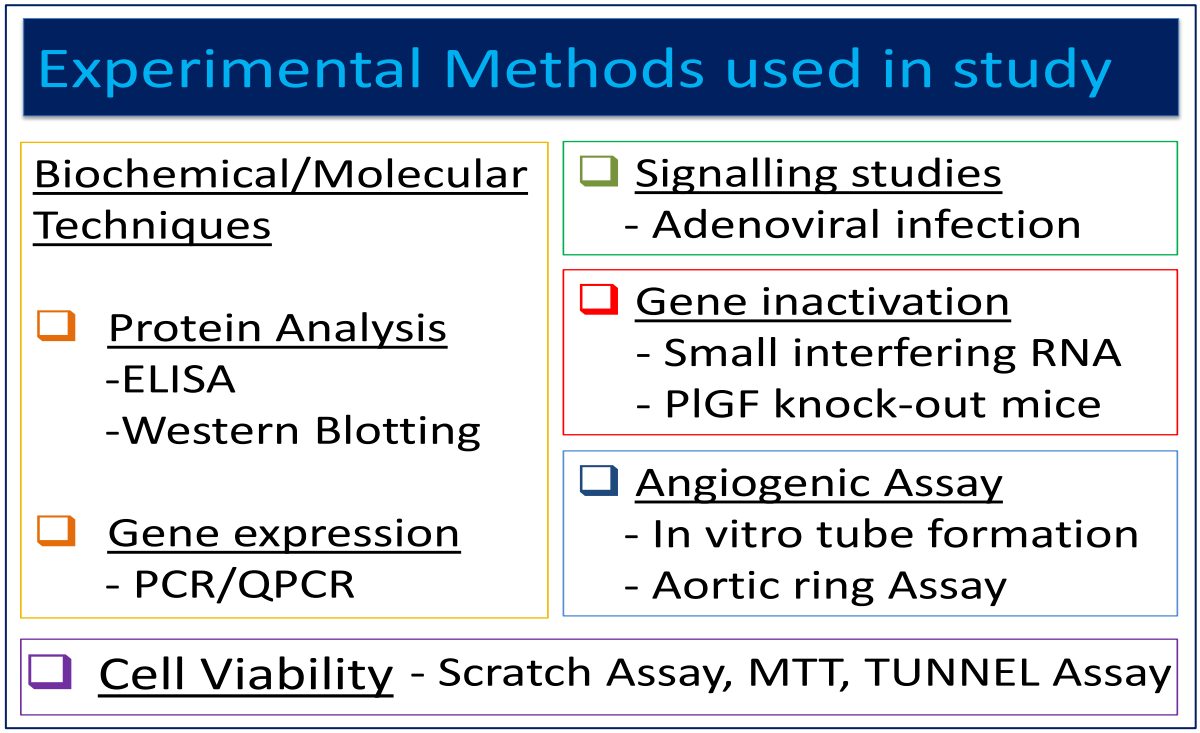


Figure 3.1 Experimental methods used in the current study. *The methods are grouped according to the analysis required, listing out the method or technique used to achieve the functions exhibited by the cells.*

3.2 Tissue and cell culture

All cell and tissue culture media including supplements were purchased as sterile or filtered through 0.22 µm pore filter prior to use. Distilled water and Dulbecco's calcium and magnesium-free Phosphate Buffer Saline (PBS) were autoclaved before use. Tissue culture plastic equipment was purchased sterile. Glassware used was washed and autoclaved prior to use. Sterile media was stored at $4^{o}C$ and used within 3 months.

3.2.1 Growth medium and supplements for cell culture

3.2.1.1 Composition of complete growth media for monolayer cells

Roswell Park Memorial Institute (RPMI)1640 medium supplemented with 10% Fetal Bovine Serum (FBS) [for HUVEC, HDMEC or HMEC-1] or HAMS F-12, 10% FBS [Cancer cell lines]. All media were supplemented with 2mM L-Glutamine, 10 U/ml penicillin, 0.1 g/ml streptomycin, which was stored at $-20^{o}C$ and used within 3 months' time.

3.2.2 Cultivation and maintenance of cell cultures

Routine maintenance of cell cultures was carried out in a Class II cabinet. Cell cultures were maintained in humidified incubators in an atmosphere of 95% air, 5% CO_2 at $37^{o}C$. Cells were seeded in 80 cm^2 tissue culture flasks in specific growth medium, and the medium was changed every 2-3 days. On reaching confluence, cells were sub-cultured by aspiration of the medium from the monolayer, followed by two washes with 5 ml PBS to remove serum. Cells were detached by treating the cells with trypsin/ Ethylene diamine tetra-acetic acid (EDTA) incubations of 2-4 minutes. To inactivate the trypsin, 5 ml of the specific growth medium supplemented with FBS was added. The cell suspension was transferred to a 15 ml conical tube and subjected to centrifugation at 80 g for 5 minutes. The supernatant was aspirated, and

the pellet suspended in 10 ml of growth medium. The cells were either split into new tissue culture flasks to maintain the stocks, or counted by haemocytometer in preparation for experiments requiring a specific number of cells per well before seeding either in T-75 cm^2 or 6, 12, 24 or 96 well tissue culture plates as per requirement. Details of cell seeding densities for human umbilical vein ECs (HUVEC) and human microvascular ECs (HMEC-1), the most commonly used cell types in this study, are mentioned in the figure legends below each of the experimental results. Each time the cells were detached with trypsin/EDTA and replated, they were assigned an ascending passage number. Table 3.1 illustrates the other cell types used in this study that was either a gift or purchased commercially.

3.2.3 Isolation and maintenance of Primary Cell Culture and Cell lines

3.2.3.1 Isolation of Human umbilical vein ECs [HUVECS]

HUVEC were isolated from the veins of umbilical cords by collagenase type-1 digestion as described elsewhere [1]. Umbilical cords were obtained from term deliveries that occurred in the Birmingham Women's Hospital NHS Trust. These umbilical cords were washed with PBS, and a 15-20 cm long section with no clamp trauma was excised. The umbilical vein in the cord was identified and cannulated with a blunt needle at one end. The other end was clamped prior to flushing the vein with PBS. This process of flushing the vein was repeated 2-3 times to ensure the removal of any blood clots. The distal end of the cord was cannulated and clamped, followed by filling up of the vein by injection of 20 ml of type-1 collagenase [1 mg/ml] made in Hank's buffered saline solution [HBSS]. The prepared umbilical cord was incubated at 37^o C for 10 minutes for the detachment of the ECs. Detached HUVECs in the type-1 collagenase solution were collected by flushing Medium 199 [M199], buffered with

Earles salt's, supplemented with 2 mM L-Glutamine, 10 mg/ml streptomycin, 100 U/ml penicillin, 20 ng/ml Epidermal Growth Factor [EGF], 2.5 ng/ml FGF-2, 20% FBS into a sterile 50 ml tube. The cell suspension was centrifuged at 80 g for 5 minutes. Supernatant was discarded and the cell pellet re-suspended in 10 ml of M199 supplemented with 20% FBS. Cells were then seeded into a 1% gelatin pre-coated tissue culture flask. The flask is placed overnight in a humidified incubator with 37 ^{o}C, and an atmosphere of 95% air, 5% CO_2 to allow the cells to attach. In order to remove erythrocytes, the medium was replaced on the following day, and the cells were grown to confluence. To assess and confirm the EC characteristics, immunofluorescence for the human von-Willebrand [vWf] factor was employed. Cells for experimental usage were between passage 2 and 6.

3.2.4 Culturing of Human Microvascular ECs [HMEC-1]

HMEC-1, a dermal microvascular cell line [a kind gift from Dr E Ades, Centers for Disease Control, Atlanta, GA] were cultured as previously described in detail (Wellicome, Thornhill et al. 1990). HMEC-1]. These were grown to confluence in T75 flasks in RPMI-1640 supplemented with 2 mM L-Glutamine, 10 mg/ml streptomycin, 100 U/ml penicillin, 20 ng/ml Epidermal Growth Factor [EGF], 2.5 ng/ml, 10% FBS and subcultured at a density of 1.5 x 10^5 on 24 well plates. Cells, on reaching confluence, were serum deprived in RPMI-1640 medium containing 0.5% FBS overnight for subsequent experimental use.

3.2.5 Cancer cell lines

Cancer cell lines were subcultured in RPMI 1640 medium containing 10% FBS and plated at a density of 1.5x10^5 cells/well. Prior to the day of conducting the experiment, cells were serum deprived in RPMI 1640 medium containing 0.5% FBS overnight. Cell supernatants

were collected and centrifuged at 85 g before storage at -80^{o}C. Cancer cell lines used in the study are listed in Table 3.1

Source	Cell Name	Tumour type	Reference
Mammary Gland; Breast	MDA-MB-231	Adenocarcinoma	(Cailleau et al., 1974)
	MCF-7	Invasive Breast Ductal carcinoma	(Soule et al., 1973)
	BT-20	Invasive ductal carcinoma	(Lasfargues &Ozzello,1958)
	T-47D	Invasive ductal carcino	(Keydar et al., 1979)
Small Cell Lung Carcinoma	N417A	Cancer Cell line	(Kiefer et al., 1987)
	N592	Cancer Cell line	(Kiefer et al., 1987)
	N249	Cancer Cell line	(Pettengill et al., 1980)
Colon Carcinoma	HT29	Adenocarcinoma	(Fogh et al., 1979)
Choriocarcinoma	JAR	Trophoblastic tumor of	(Kameya et al., 1975)

Table 3.1 List of cancer cell source and cell types used in this study.

3.2.6 Cell Cryopreservation

A reserve stock of the cells was maintained through cryopreservation. Confluent monolayers of cells to be preserved were washed twice with PBS and trypsinised for 2-4 minutes, following the addition of 5 ml of complete medium. The cell suspension was centrifuged at 80 g for 5 minutes, and the pellet was suspended in 1 ml of pre-cooled culture medium containing 10% FBS and 10% Dimethyl Sulphoxide [DMSO]. The cell suspension was transferred to sterile cryovials. The vials were labelled with cell type, passage number, and date of storage. Freezing of the cells was carried out in Mr. Frosty at a rate of –1^{o}C/minute in 100% isopropyl alcohol over a 4 hour period. The frozen vials were then transferred to racks in at –70^{o}C and catalogued.

3.2.7 Cryopreserved cell retrieval

To retrieve the preserved cells, the cryo vials were thawed to 37^{o}C incubated at room temperature. Once thawed, the cell suspension was immediately transferred to a tissue culture flask with specified serum-containing growth medium. Cells were incubated at 37^{o}C in a humidified incubator in an atmosphere of 95% air, 5% CO_2 for about 16 hours. This enabled the cells to attach to the flask. The cells were then washed with PBS to remove traces of DMSO and cell debris, and replaced with fresh growth medium.

3.2 Stimulation of ECs

HMEC-1 or HUVEC were cultured in 24 well cell culture plates at a density of 1.5x10^{5}cells/well and incubated at 37^{o}C until they reached confluence. The mean time to achieve confluence was 24 to 30 hours and was dependent on passage number of the cells. Confluent cells were starved in appropriate growth medium overnight. Cells were stimulated in duplicate with various growth factors or inhibitors or both. All stimulations were performed in 500 µl for 24 hours unless otherwise indicated. Supernatants were collected and centrifuged at 85 x g before storage at -80^{o}C.

3.2.1 Analysis of Changes in Intracellular Signalling Pathways

Various pharmacological inhibitors and activators were used to elucidate the mechanism of FGF-2 or TNF-α-mediated PlGF release from HMEC-1 or HUVEC. Confluent cells in 6 well cell culture plates at a density of 1 x 10^{6}cells/well in 2 ml/well of the growth medium.

Pharmacological inhibitors or activators were added 30-45 minutes before the treatment with FGF-2. At indicated time points cells were aspirated of growth medium and washed twice with 10 ml of ice-cold PBS/flask. Cells were subsequently subjected to lysis treating with 60 µl/well of non-ionic radio immunoprecipitation buffer [RIPA] containing 1:1000 dilutions of

protease inhibitor and phosphatase inhibitors I and II. A sterile cell scraper was used to harvest the cell lysates which were collected into 0.5 ml eppendorf tubes. Duplicate samples were pooled to generate sufficient amounts of protein and allow ample protein for Western blotting. Samples were stored at -80^{o}C for 1 hour and then thawed on ice before being centrifuged at 1300 rpm for 10 minutes to remove any insoluble proteins that may have been present. Supernatants were aspirated, and the pellet was re-suspended in 50 ul of 1x RIPA buffer and assay for protein concentration.

3.3 Adenoviruses

3.3.1 Adenoviral infection of ECs

HMEC-1 at 90% confluence were infected with the adenoviruses for a minimum of 12-16 hours in M199 containing 5 % FBS and G/P/S at 37 °C. Adenovirus containing medium was removed and replaced with fresh growth medium for a further 24 hours. Cells were then cultivated in M199 containing 5 % FBS and L-Glutamine, 10U/ml penicillin and 0.1 μg/ml streptomycin (G/P/S) for the appropriate time periods. Infection was validated by subjecting protein lysates of infected HMEC-1 to SDS-PAGE, and then Western blotted with appropriate antibodies diluted in TBS-T/0.5 % BSA (see Appendix II for antibody source and dilution used for Western blotting).

PROTEIN BIOCHEMISTRY TECHNIQUES

3.4 General ELISA Protocol

Nunc-Immuno™ MicroWell™ 96 well solid plates were coated with 100 μl/well of capture antibody in PBS pH 7.4, covered with a plastic film and left in the dark overnight at room temperature (RT). The following day the plates were washed four times with PBS containing

0.05 % Tween [PBS-T] and then blocked for 1 hour using 200 µl/well of 1 % BSA in PBS. After three PBS-T washes to remove residual BSA, samples were mixed well and added at 100 µl/well to the plates followed by incubation for 2 hours with agitation at RT. After washing, 100 µl of biotinylated detection antibody constituted to working concentrations in PBS-T was added to each well and incubated for a further two hours at RT with agitation. After washing streptavidin-HRP [1:200] in PBS-T was added for 20 minutes in the dark and then washed off. The Hydrogen peroxide-colour substrate solution [R&D Systems, UK] was added to the plate [see manufacturer's instructions], incubated until sufficient colour change was observed and the reaction stopped using 2N H_2SO_4. Presence or absence of the protein of interest was determined by reading the optical density [OD] at 450 nm [adjusted at 540 nm] with a Multiskan Ascent 96 well plate reader and subtracting the blank value [sample diluents only] from the sample absorbance values.

3.5 PlGF or VEGF ELISA

Supernatants from HUVEC, HMEC-1 or cancer cell lines were incubated on 96 well plates and were coated with mouse anti-human PlGF or VEGF capture antibody in PBS [pH 7.4] [R&D Systems, UK]. 100 µl of recombinant human PlGF or VEGF standard in assay buffer [range 1- 2000 pg/ml] or cell supernatants were added and incubated at room temperature for 2 hours. PlGF or VEGF release was detected with 100 µl/well biotinylated mouse anti-human PlGF or VEGF detection antibody for 2 hours [0.4 µg/ml] [R&D Systems, UK] and detected as described above.

PlGF standards or cell supernatants were pre-incubated with increasing concentrations of sVEGFR-1 [0.01 to 100], to ensure that the R&D PlGF ELISA kit measured total PlGF. No

significant effect was seen on the level of PlGF measured in the ELISA indicating that both the sVEGFR-1 bound and free PlGF protein is being detected.

3.6 Protein extraction

The day before stimulation or transfection cells were fed with full media and seeded at a density of 5 $x10^5$ cells/ml. Usually, ~ 5 $x10^6$ cells [roughly 10 ml of cell suspension] were harvested and washed three times with 10 ml of cold PBS, centrifuging for 7 min at 1500 rpm and 4^oC. The final wash was carried out in 1.5 ml Eppendorf, after the last wash all supernatant was aspirated on ice. The cell pellet was then either snap-frozen in liquid nitrogen and stored at -80^oC for future use or lysed in between 50-500 µl of lysis buffer [9M urea, 50 mM Tris pH7.5. 15 mM β-mercaptoethanol], unless otherwise stated. Following addition of lysis buffer, samples were vortexed vigorously for 1 min and then clarified by centrifugation

3.6.1 Protein Quantification

The protein concentration of the samples was quantified by the Bradford method of protein assay for equal loading of the samples on a Western blot gel. A protein standard curve was made using BSA standard protein [range from 0 - 1.4 mg/ml] dissolved in RIPA buffer. It was important that both the buffer with which the standard curve was created and used to re-suspend the protein samples were identical. Thus, differences in protein concentrations observed between the samples were due to sample protein content and could not be attributed to buffer related artefacts. Protein samples were created in RIPA buffer to enable protein detection within the range of the standard curve. Following dilution, 5 µl/well of samples and standards were added in duplicate wells to 96 well plates followed by 25 µl/well of Bio-Rad Solution A and 200 µl/well of Bio-Rad Solution B. Plates were incubated for 15 minutes at room temperature before the optical absorbance was read at a wavelength of 595 nm with a

Thermo Lab Systems MultiScan Ascent microplate photometer. Optical absorbance values for protein samples were used to extrapolate protein concentration from the BSA standard curve. Protein samples loaded for the Western blots were equal in concentration as well as volume; this was achieved by diluting the sample with 2 x sample buffer to ensure equal loading of protein content and equalise volumes of each sample prior to applying onto the gel, to avoid lane artefacts. This step ensured that all of the samples were treated under identical conditions in the gel and any differences in protein expression observed by Western blotting are attributable to the changes in EC function rather than to gel loading or protein transfer errors.

3.7 Western Blotting

3.7.1 Polyacrylamide Gel Preparation and Running

To separate the samples by SDS-PAGE, the appropriate percentage of running gel was prepared that was based on the size of proteins to be detected. The recipes for different percentage of running gel are listed in the appendix. After the suitable running gel had been made, the mixture was immediately pipetted between two securely fastened glass plates up to a predefined mark determined by the height of the loading comb. A 50% methanol solution was applied immediately above this layer to prevent the formation of air bubbles in the running gel, and to generate a level running gel-stacking gel interface to ensure the samples would run in a parallel fashion. The gel was allowed to polymerise for 30 minutes before the methanol was removed and was replaced with 5% acrylamide stacking gel [2.7 ml 40% Acrl/Bis, 0.5 M Tris [pH 8.8] 200µl 10% SDS, 100µl 10% APS, 30µl TEMED and 24ml water] within which the loading comb was inserted. The stacking gel was given another 15 minutes to polymerise before the apparatus was secured into the gel running tank. The tank was then filled with 1x running buffer [0.05 M Tris, 0.384 M glycine and 3.47 mM SDS] to

prevent the gel from drying out. Samples were boiled, cooled on ice and then centrifuged at 1300 rpm for 1 minute. Samples were loaded into the lanes in accordance with a pre-prepared plan so that each lane had a maximal protein concentration of 20 ug/ml, 7 ul/lane of rainbow marker, plus 5 ul/lane of biotinylated marker were also boiled with 2 x sample buffer as described above and added to lanes flanking the samples. 2 x sample buffer was used to fill sample-free lanes to ensure that the sample buffer itself was not contaminated with protein. The gel was run for 30 minutes at 60 V and then run for a further 90 minutes at 110 V once samples had migrated from the stacking gel into the running gel.

3.7.2 Semi-Dry Transfer of proteins onto Nitrocellulose Membrane

Following separation, the gel was removed from the glass plates. The stacking gel and excess running gel were removed before the remaining gel was immersed in transfer buffer [5.81g Tris, 0.375 g SDS, 2.93 g Glycine, 200 ml 20% methanol and 800 ml dH2O]. Nitrocellulose membranes and stacking filter paper were cut to appropriate sizes and soaked in transfer buffer. The filter paper was placed in the transfer buffer upon which the nitrocellulose membrane, running gel and second filter paper were stacked [in that order]. Air bubbles were removed by rolling the layers with a pipette. Protein transfer was run for 2 hours at a constant current of 0.8 mA/ cm^2].

3.7.3 Blocking Non-Specific Binding Sites on the Nitrocellulose Membrane

Blocking the membrane prevents non-specific protein binding during subsequent stages of membrane handling and reducing non-specific binding of the primary and secondary antibodies. Following transfer, filter papers and gels were discarded while the nitrocellulose membrane was blocked in a blocking buffer consisting of 8% fat-free skimmed milk powder in TBS-T [20 mM Tris, 73.1 mM NaCl, 0.1% Tween-20® [pH 7.4]]. After 1 hour, the

blocking buffer was removed, and membranes were washed twice in TBS-T. By this stage, the non-specific milk proteins had bound to all available binding sites on the nitrocellulose membrane devoid of sample protein.

3.7.4 Primary and Secondary Antibody

Primary antibodies were diluted in 5% BSA solution containing sodium azide which prevents microbial growth in the nutrient-rich buffer. Primary antibodies were all used at a dilution listed in Table 3.2 and were raised in rabbit or mouse [except β-actin which was used at a dilution of 1:2000 and raised in mouse]. Membranes were probed with anti-total Erk1/2, phospho Erk1/2, c-Raf, β-actin or pan PKC primary antibody overnight at 4^{o}C.

Antibody	Species raised in	Dilution for WB	Source
β actin	mouse	1:20,000	Cell Signaling Tech, Herts, UK
Akt	rabbit	1:1000	Cell Signaling Tech, Herts, UK
Akt [phospho ser-473]	rabbit	1:500	Cell Signaling Tech, Herts, UK
ERK1/2	rabbit	1:1000	Cell Signaling Tech, Herts, UK
ERK1/2 [phospho thr-202/tyr204]	rabbit	1:1000	Cell Signaling Tech, Herts, UK
His-Tag	mouse	1:1000	Autogen Bioclear, UK
PLCγ [phospho tyr-783]	rabbit	1:1000	Cell Signaling Tech, Herts, UK
PlGF [172]	rabbit	1:100	Gift – H. Weich
RAS	mouse	1:500	BD Transduction Labs, UK

Table 3.2 Antibodies used in Western Blotting for protein detection

The following day, membranes were washed three times for 20 minutes each in TBS-T and were incubated with horseradish peroxidase [HRP] labelled secondary antibody or β-actin] at a 1:5000 dilution in 5% (w/v) skimmed milk powder in TBS-T. Table 3.3 illustrates the antibodies used for the purpose of the study, the species the antibody was raised in, the dilution used for western blotting and the source of the antibodies. A horseradish protein (HRP) conjugated biotinylated anti-biotin marker was added at a 1:1000 dilution to detect the biotinylated markers, which had also been transferred along with the sample protein to the

membrane. After 1 hour, the membranes were quickly washed twice in TBS-T before being washed a further three times for 20 minutes each in TBS-T.Secondary antibody conjugates.

Antibody	Dilution	Experiment	Source
HRP IgG conjugates	1:5000	WB	Vector Labs

Table 3.3 Secondary antibody conjugates used for Western Blotting

3.7.5 Enhanced Chemiluminescence Detection

ECL solution was made using an equal volume of SuperSignal® West Pico luminal/enhancer and SuperSignal® West Pico stable peroxidase solution [Pierce Biotechnology Inc]. Membranes were incubated in ECL for 30 minutes before being wrapped in Saran wrap and secured in an autoradiographic cassette. The membrane was opposed to Kodak X-OMAT 5 autoradiographic film for periods of time ranging from 5 minutes to 20 seconds in the dark room. The exposed film was first immersed in developer solution [Kodak] for 90 seconds and was secondly immersed in fixer solution [Kodak] before being washed with water and air-dried. An automated film developing system was used in later experiments.

3.7.6 Protocol for Stripping and Re-probing the membrane

Membranes were immersed in stripping buffer [1.97 g Tris HCL [pH 6.7], 4 g SDS, and 1.4 ml 2- mercaptoethanol and 200 ml dH_2O] for 60 minutes at 30 ^oC to remove the membrane-bound primary and secondary antibodies. Membranes were then washed twice for 10 minutes in TBS-T followed by a further 3 washes for 20 minutes each in TBS-T. Membranes were re-blocked in 10% milk before the protocol from using the primary antibody was repeated.

3.8 Cell Viability by MTT assay

To ensure that the cytokines and signalling inhibitors used in cell-based assays were not affecting cell viability [3-[4, 5-Dimethylthiazol–2-yl]-2, 5-diphenyltetrazolium bromide thizolyl blue] (MTT) assay was performed. MTT assay is a colorimetric assay that measures the mitochondrial activity, and the total mitochondrial activity is related to the number of viable cells. This assay is broadly used to measure and detect any *in vitro* cytotoxic effects on cells (van Meerloo, Kaspers et al. 2011).

The cell viability in this assay is based on the conversion of MTT, a tetrazolium salt, to an insoluble formazan product. This is largely achieved through the action of mitochondrial dehydrogenase enzymes in metabolically active cells. Cells were seeded at a density of 1.5 x 10^4 cells/well in 96-well plates, left to attach to the plate for 4 hours and then rested overnight in medium containing 2% FBS. The resting medium was aspirated from the cells and replaced with 100 µl of fresh 2% FBS medium containing the growth factors at appropriate concentrations. After 24 hour incubation at 37 °C, the growth factors were removed and 75 µl of fresh 2% FBS medium was added. 25 µl of MTT at a concentration of 5 mg ml^{-1} was added to each well and the plates were incubated in the dark at 37 °C for 4 hours. MTT reagent was prepared by adding 3 ml of MTT to 9 ml of 10% FBS RPMI-1640. 100 µl of MTT reagent at a concentration of 5 mg/ml was added to each well following aspiration of conditioned media and HUVECs were incubated in the dark for 4 hours at 37 ^oC and 5% CO_2. After removing the medium, 150 µl of 99.5% Dimethyl Sulphoxide [DMSO] was added to each well to solubilise the crystalline formazan and thus changes the shape of the absorbance spectrum of the solution to enable quantification by spectrophotometric analysis. Optical absorbance was

read at 540 nm with the Thermo LabSystems MultiScan Ascent microplate photometer and analysed.

3.9 Molecular Biology techniques

3.9.1 Total RNA Extraction

RNA was extracted following stimulation of cells using the RNA Easy Mini Kit [Qiagen] according to the manufacturer's instructions. The concentration and quality of RNA were assessed using the Nanodrop spectrometer. The $A_{260/280}$ of RNA samples was required to be approximately 1.8. RNA stocks were diluted to 1 mg/ml for routine use. All RNA samples were stored at –80^{o}C.

3.9.2 cDNA synthesis

The extracted RNA was subjected to digestion using DNase-1 enzyme on RNeasy mini columns [Qiagen]. Using the cDNA Synthesis Kit [Bioline], the total RNA [1 µg] was reverse transcribed with oligo-dT18 primers for 70 minutes at 48°C.

Real-time PCR on a Corbett Research Rotagene RG-3000 was performed on the samples using SYBR green PCR SensiMix [Bioline] at 95°C, 10 seconds; 58°C, 15 seconds; 72°C, 15 seconds. The real-time PCR machine was programmed to quantify fluorescence after 82°C, 15 seconds, extended to 40 cycles. Using primers specific for human PlGF [sense, 5´-GCGATGAGAATCTGCACTGT-3´; antisense, 5´-CTTTAGGAGCTGCATGGTGA-3´] or β-actin, cDNA samples and their standards were amplified as previously described (Malarstig, Tenno et al. 2003)(R&D systems).

Gene Primer	Sequence	Source
hβ-actin	5'-TCACCCACACTGTGCCCATCTACGA-3' 5'-CAGCGGAACCGCTCATTGCCAATGG-3'	(Malarstig, Tenno et al. 2003)
hPlGF	5'-GCGATGAGAATCTGCACTGT-3' 5'-CTTTAGGAGCTGCATGGTGA-3'	In-house designed using Primer 3 Software (http://frodo.wi.mit.edu/primer3)

Utilising the plasmid containing a C-terminal fragment of PlGF or β-actin cDNA, the mean threshold cycle (Farina, Sekizawa et al. 2008) for each sample was compared with standard curves produced using serial dilutions, to ensure that the primers could detect the targets over a large concentration range and to determine the detection limit/sensitivity as well. Relative PlGF expression was calculated following normalization using β-actin Ct value for the same sample. The Ct value for every gene of interest normalised using the β-actin Ct value for the same sample. Change in fold change calculated using the equation $2^{-\Delta\Delta ct}$.

$\Delta\Delta Ct$ = (ΔCt value of the treated cells) – (ΔCt value for the control)

3.10 Gene Inactivation Studies

PlGF gene silencing studies were carried out by small RNA interference [sRNAi] by Amaxa for assessing PlGF gene function in HUVEC. PlGF ELISA assessed the degree of transfection by PlGF knock-down in HUVEC.

3.10.1 Nucleofection

To knock-down PlGF gene expression, HUVEC were transfected separately with PlGF siRNA [forward 5'-CCGGCUCGUGUAUUUAUUAUU-3'; reverse 5'-AUAAAUACACGAGCCGGUU-3'], or control siRNA which does not target any sequence in the human genome. The nucleofection technique is an electroporation based transfection technique. The day before, transfection cells were fed with complete medium and seeded at a density of 1.5 x 10^5 cells/ml. HMEC-1 or HUVEC were transfected using Nucleofection Cell Line Kit T and a Nucleofector electroporator according to the manufacturer's instructions (AMAXA, Germany). 2 x 10^6 cells were used per transfection. Cells were transfected with 5 ug of siRNA using appropriate cell type-specific programme for HMEC-1 or HUVEC on the Nucleofector. Following transfection, cells were immediately transferred to a 12-well tissue culture plate and incubated in 1.5 ml of complete medium for 8 hours. Cells were subsequently diluted to 5x10^5 cells/ml and cultivated to be used for cell function assays.

3.11 Functional Assays

3.11.1 Tunel Assay – Dead End Fluorometric Assay

3.11.1.2 Assay Principle

Dead End Fluorometric assay was used to enable precise detection of apoptosis in situ in PlGF silenced HUVEC (PlGF siRNA) cells when compared to their respective control (control siRNA). The principle underlying this assay is the detection of DNA fragmentation in the apoptotic cells. Recombinant Terminal Deoxynucleotidyl Transferase, enzyme (rTdT) measures the fragmented DNA of apoptotic cells by catalytically incorporating labelled dUPT at 30 -OH DNA fragment ends (Darzynkiewicz, Galkowski et al. 2008).

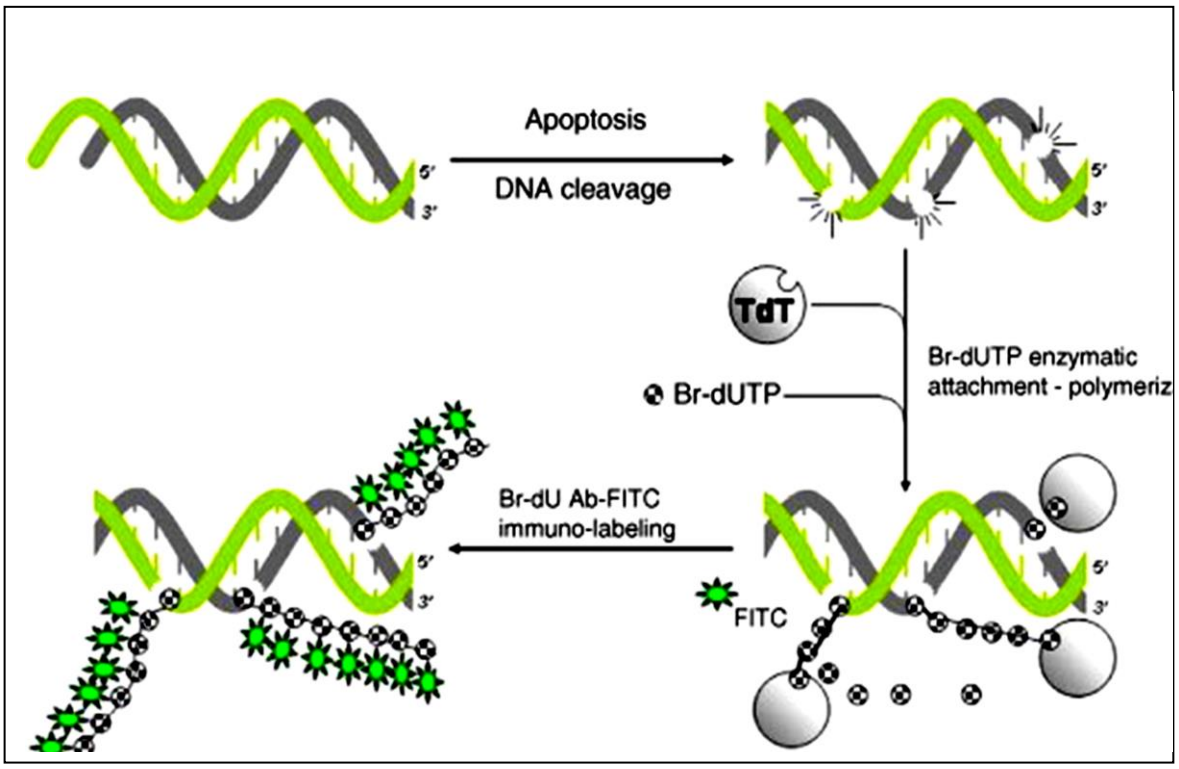


Figure 3.2 Schematic illustration of DNA strand breaks labelling for TUNEL assay *with Br-dUTP utilising exogenous terminal deoxynucleotidyl transferase (TdT) (:http//www.google.com/images).*

3.11.1.3 Protocol

HUVEC (control siRNA) and PlGF silenced HUVEC (PlGF siRNA) were plated at a density of $1x10^5$ on a poly-l-lysine-coated two-well microscope chamber slides for 6 or 12 hours. Instructions for performing the assay were followed according to the manufacturers' instructions (Promega). Cells were then fixed in 4% methanol free formaldehyde and subjected to the DeadEnd™ Fluorometric TUNEL System. This involved labelling of DNA strand breaks with fluorescein-12-dUTP for 60 minutes. Samples were immediately analysed using a fluorescence microscope. Protocol flow chart provided in figure 3.3.

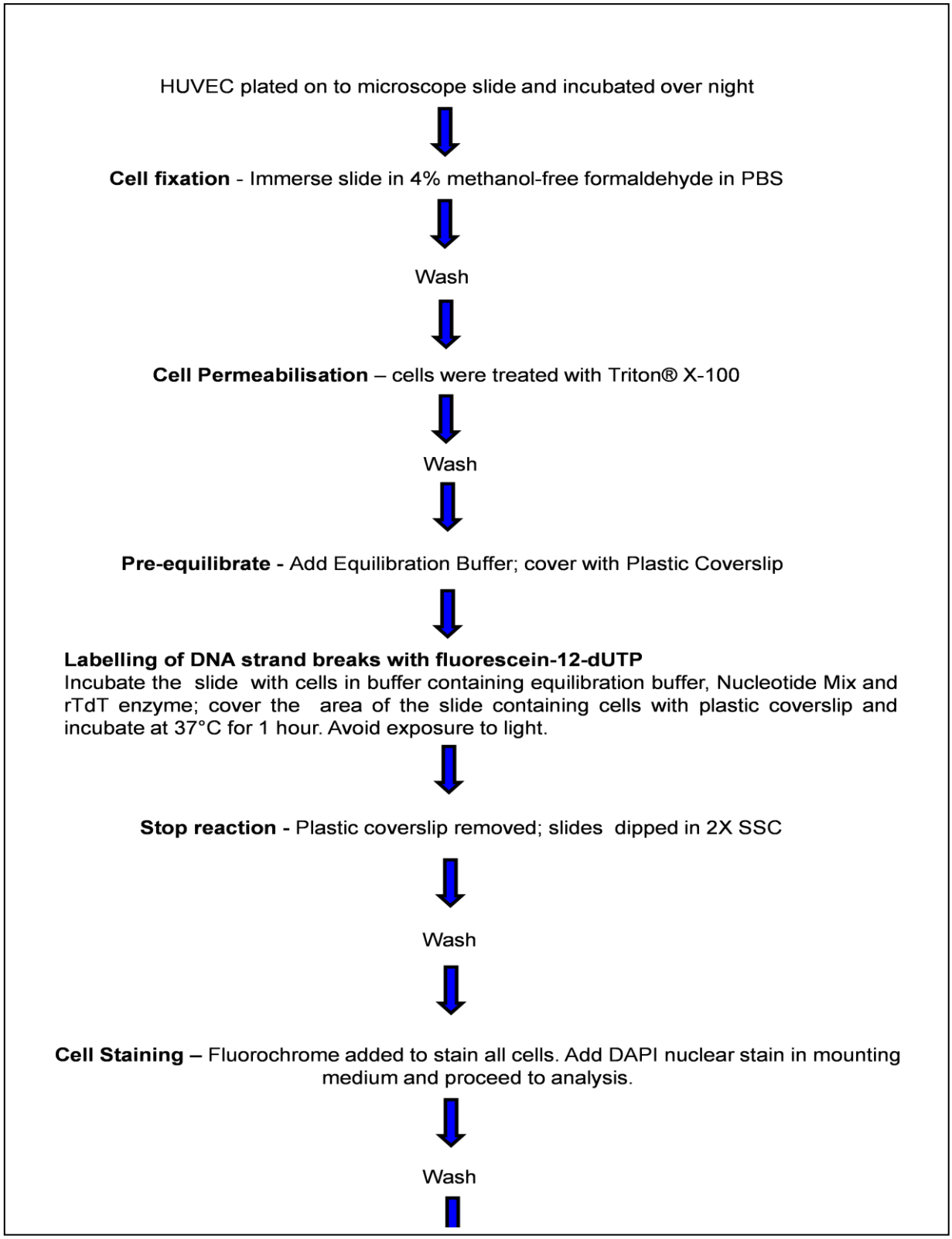


Figure 3.3 Protocol overview of the DeadEnd™ Fluorometric TUNEL System used to detect cell apoptosis.

3.12 In Vitro Tube Formation

Formation of tubular structures was studied using HUVEC cultured on growth factor-reduced Matrigel diluted 1:1 in ice-cold M199 as previously described (Ahmad and Ahmed 2004). Briefly, cells were seeded onto the Matrigel for 45 minutes and treated with FGF-2 (2 or 10 ng/ml). After incubation at various time points, cells were observed under a Nikon inverted microscope and results recorded at times specified using Image-Pro Plus image analysis software (Media Cybernetics). Mean total tube length was calculated from three independent experiments.

3.12.1 Animal Preparation

All procedures involving mice were performed according to the protocols approved by the University of Birmingham. Mice between 8-10 weeks of age for wild-type (WT) and PlGF-KO mice on a Balb/c background were kindly provided by [redacted] at the [redacted]. Validation of the PlGF-KO mice was carried out in the laboratories of [redacted].

3.13 Aortic Ring Assay

The aortic ring assay was performed as previously described (Blacher, Devy et al. 2001). Briefly, aortic explants were resected from PlGF-deficient mice (PlGF-KO) or wild-type (WT) mice. The descending thoracic aorta was very carefully isolated, then 1mm length aortic rings were cut and embedded into growth factor-reduced Matrigel and collagen mix, supplemented with 20 U/mL heparin on 48 well plate. The explant resections were treated with FGF-2 (2 or 10 ng/ml). Each experimental condition on the explant resections was performed in duplicates. 2.5 % of mouse serum was added to each condition (Carmeliet,

Moons et al. 2001). Aortic ring assay was performed with three biological replicates (n=3) and six technical replicates (n=6).

Explant cultures were examined every second day. On day seven, vessels sprouting from aortic explants were examined using an inverted phase-contrast microscope. Results were recorded at times specified using Image-Pro Plus image analysis software (Media Cybernetics). Quantification was achieved by measurement of the length and number of vessel-like extensions from the explants.

3.14 Statistical Analysis

The data in this thesis are expressed as mean [± Standard Error] of minimum 3 independent experiments unless otherwise indicated. A comparison between controls and samples and between individual samples was made with the use of a two-tailed unpaired Student's t-test with a 95% confidence interval. $P<0.05$ was considered to be significant. All statistical analyses were performed using Graph Pad software version Prism.

Chapter 4 -Growth factors and cytokines regulating endothelial PlGF levels

4.1 Introduction

Development and growth following normal physiology depends primarily on oxygen and nutrients provided by blood vessels. Consequently, malfunctioning or dysfunction of these blood vessels contributes towards compromised organ function, leading to congenital and acquired diseases. Advancements of therapeutic strategies to either promote revascularisation of ischemic tissues, or to inhibit angiogenesis in cancer and inflammatory disorders with the goal to combat diseases have been a prime focus for over a decade. Administration of recombinant VEGF or anti-VEGF therapies (Losordo, Vale et al. 1998, Kastrup, Jorgensen et al. 2005, Stewart, Hilton et al. 2006), have been in use since 2004, with its potential risks/side effects such as hypotension, bleeding, oedema formation (Yang, Bunting et al. 2000) and possible risk of inducing neoplastic and malignant disease (Lee, Springer et al. 2000) out weighing its benefits.

PlGF, a VEGF homolog and a key regulator in pathological angiogenesis (Carmeliet, Moons et al. 2001, Luttun, Tjwa et al. 2002), is detected in plasma and tumours, correlating to tumour staging, vascularity, recurrence, metastasis and in the survival of these tumours (Matsumoto, Suzuki et al. 2003, Chen, Hsieh et al. 2004, Parr, Watkins et al. 2005, Wei, Tsao et al. 2005, Ho, Chen et al. 2007). Anti-PlGF treatment both *in vitro* and mouse *in vivo* studies demonstrated inhibition of growth and metastasis in various tumours, including those resistant to VEGF[R] inhibitors [VEGF^{R}Is] (Fischer, Jonckx et al. 2007). This study also ascertains the inhibitory effect of anti-PlGF on both angiogenesis and lymphangiogenesis, with a distinct feature of preventing angiogenic rescue program, with efficacy and safety unlike VEGF^{R}Is therapies (Fischer, Jonckx et al. 2007). Elevated levels of PlGF have also been reported in several inflammatory disorders. PlGF has been reported to be indispensable for physiological vascular development (Carmeliet, Moons et al. 2001). The role of PlGF on proliferation,

migration and permeability of ECs has been controversial (Park, Chen et al. 1994, Persico, Vincenti et al. 1999). Absence of PlGF induced regression of neovascular complexes, and had a minimal effect on vascular development and normal embryogenesis as demonstrated in PlGF knockout mice. Yet such a deficiency can reduce collateral vascular growth under pathologic conditions, such as ischemia, inflammation, and cancer (Carmeliet, Moons et al. 2001). PlGF stimulates collateral growth in ischemic heart and limb with an efficiency comparable to that of VEGF, without affecting healthy vessels (Pipp, Heil et al. 2003, Luttun, 2002 22), which makes PlGF the most wanted candidate to learn its functional aspects in order to manipulate as a therapeutic agent. Limited knowledge of the cell types producing PlGF and the factors regulating PlGF levels deters the efforts to combat several pro/anti angiogenic disorders. It is therefore essential to establish the source of PlGF and the factors regulating this pleiotropic cytokine.

In this study we clearly demonstrate the specific and strong expression of PlGF at both the gene and protein levels in a wide range of ECs, unlike cancer cell lines. We also screened various growth factors and cytokines to evaluate their effect in modulating the levels of PlGF secreted by ECs. This study formed the basis of the later chapters to further investigate the underlying mechanisms regulating PlGF in ECs. FGF-2 and VEGF belonging to the class II and class V receptor tyrosine kinase family [RTK] were strong inducers, in contrast to the Th1 type pro-inflammatory cytokines, such as, TNF-α, that reduced secretion of PlGF from ECs.

4.2 Results

4.2.1 PlGF is selectively expressed in Endothelium

As relatively little is known of the cell-specific regulation of PlGF expression, we screened a panel of tumour cell lines (including those derived from breast, colon and lung), as well as SMCs and ECs for VEGF and PlGF levels by ELISA [Figure 4, 4.2] and RT-PCR [Figure 4.1B].

In contrast to VEGF (Figure 4.2), PlGF expression was strikingly low or below detectable levels in most of the tumours, transformed and normal cells examined (Figure 4.1). However, high levels of PlGF protein and mRNA levels were detected in a range of human microvascular and macrovascular ECs. PlGF expression was very low or below detectable levels in most of the tumour cell lines. In contrast, high levels of PlGF mRNA and protein levels were detected in ECs, re-affirming ECs to be an abundant source of PlGF (Yonekura, Sakurai et al. 1999). SMCs demonstrated significant levels of PlGF compared to the tumour cells, but very low levels when compared to ECs.

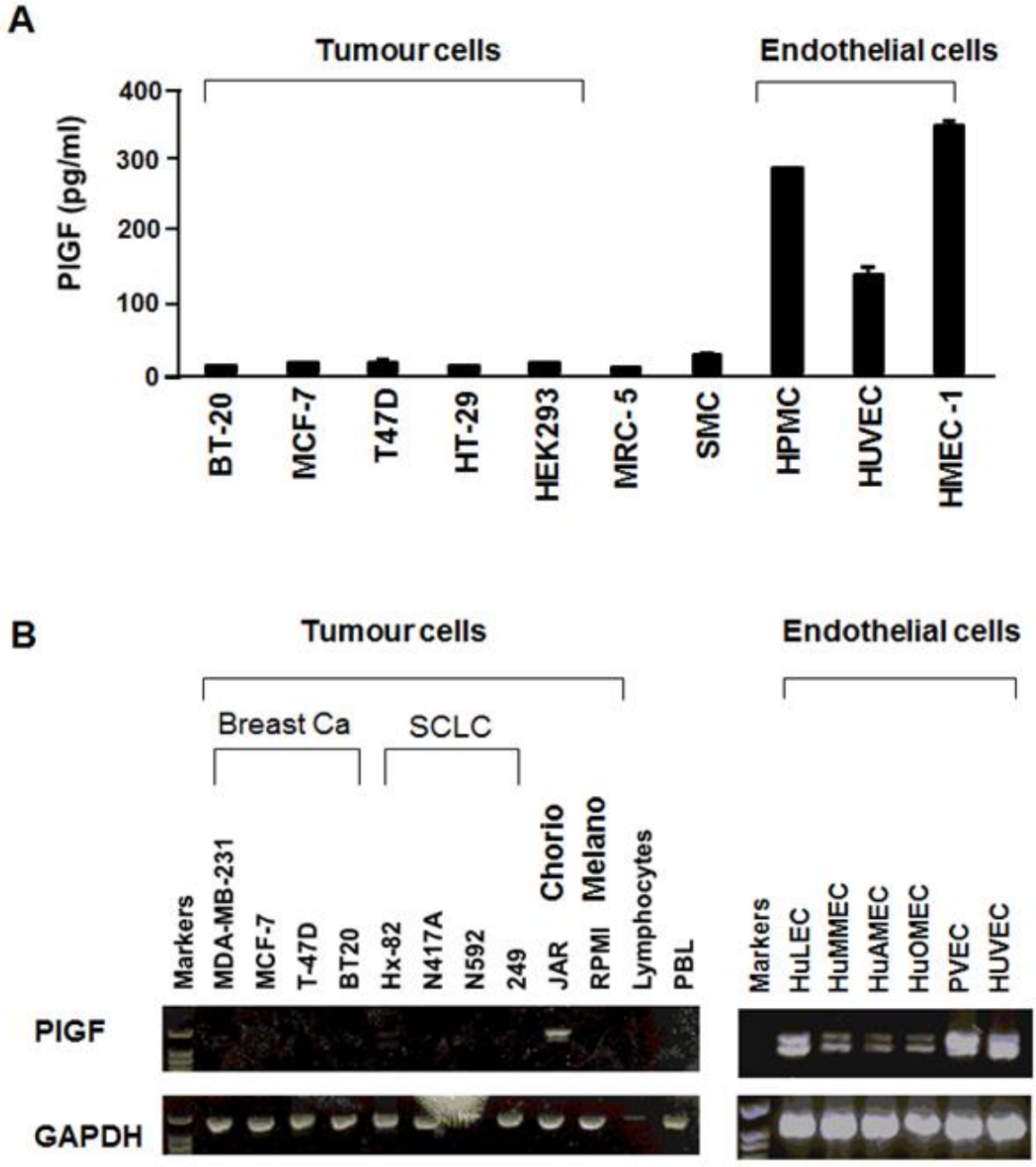


Figure 4.1 PlGF selectively expressed in the endothelial cells. *PlGF protein release by ELISA (A) and PlGF expression by RT-PCR (B) from various tumour and normal cells: human breast (MDA-MB-231, MCF-7, BT20, T-47D), small cell lung carcinoma (SCLC: Hx-82, N417A, N592, 249) colon (HT29), choriocarcinoma (JAR), melanoma (RPMI) cancer cell lines; embryonic kidney epithelial cells (HEK293); foetal lung fibroblasts (MRC-5), and aortic smooth muscle cells (HA-SMC). Human endothelial cells derived from aorta (HAEC), umbilical vein (HUVEC), pulmonary vein (PLVEC): and microvascular cells from lung (HuLEC), mammary adipose (HuMMEC), abdominal adipose (HuAMEC), Human pulmonary endothelial cells (HPMC), and the dermal microvascular endothelial cell line (HMEC-1). PlGF was measured by ELISA in cell supernatants following 24 h incubation in basal medium containing 5% FCS. (B) RT-PCR for PlGF in cancer cells and endothelial cells. Data are*

expressed in pg/ml of free and bound PlGF and are mean [± SEM] of three or more separate experiments performed in triplicates. RT-PCR data Courtesy Dr P Hewett.

Interestingly, the same samples screened for VEGF levels demonstrated diametrical results, as previously reported in thyroid tumour cell lines (Viglietto, Maglione et al. 1995). This clearly demonstrates and re-confirms that ECs are most likey to be the prime source of PlGF, which is consistent with previous reports.

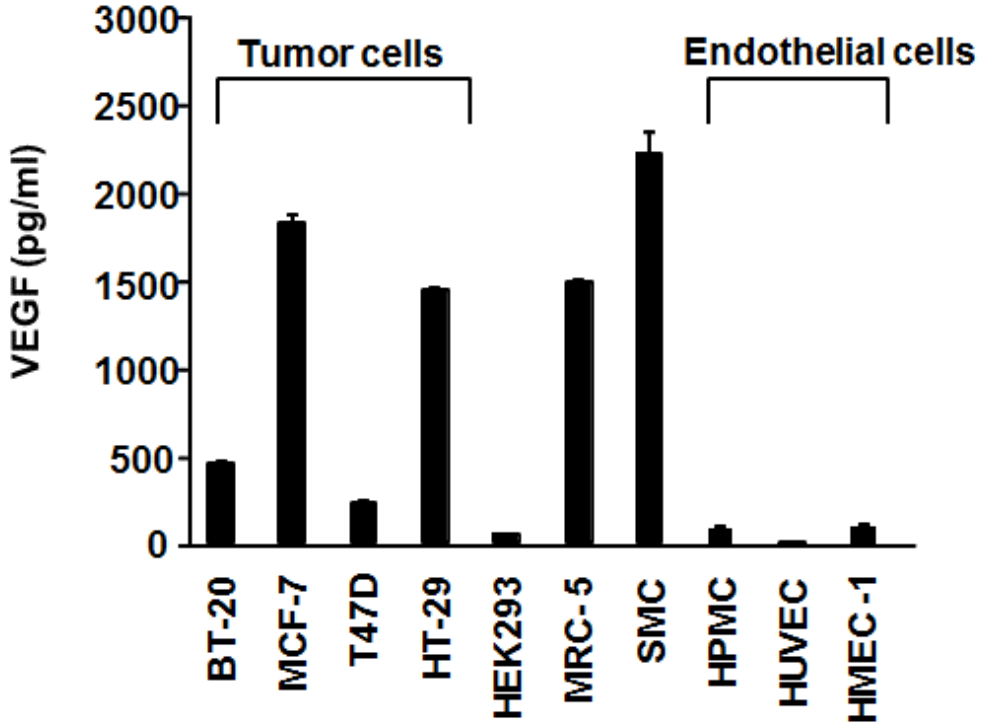


Figure 4.2 VEGF levels in endothelial and non-endothelial cells. *VEGF release from various tumour and normal cells: human breast (BT-20, MCF-7, T-47D), colon (HT29), cancer cell lines; embryonic kidney epithelial cells (HEK293); foetal lung fibroblasts (MRC-5), and aortic smooth muscle cells (SMC). Human endothelial cells derived from Human pulmonary endothelial cells (HPMC), umbilical vein (HUVEC), and the dermal microvascular endothelial cell line (HMEC-1). PlGF was measured by ELISA in cell supernatants following 24 h incubation in basal medium containing 5% FCS. Data are expressed in pg/ml of free and bound PlGF and are mean [± SEM] of three independent experiments performed in triplicates.*

4.2.2 Factors regulating the release of PlGF in cells

After confirming that ECs are the prime source of PlGF, we sorted to study the factors that regulate the release of PlGF in HMEC-1 and primary cultures of Human macrovascular endothelial cells, HUVEC, and Smooth muscle cells [SMC]. Cells were treated with a range of growth factors and cytokines at specified concentrations. PlGF levels secreted by these cells 24 hours post treatment were measured in the cell supernatants by ELISA. Figure 4.3 represents secreted PlGF levels from HUVEC [A] or HMEC-1 [B] in the presence of a range of cytokines that disclose to be agonist or antagonist of PlGF. Two–four folds increase in the levels of PlGF was observed in the presence of PMA and FGF-2 in both the cell types, compared to the control. In contrast, GM-CSF, TNF α and IL-1β had the opposite effect; inhibiting the levels of PlGF twofold.

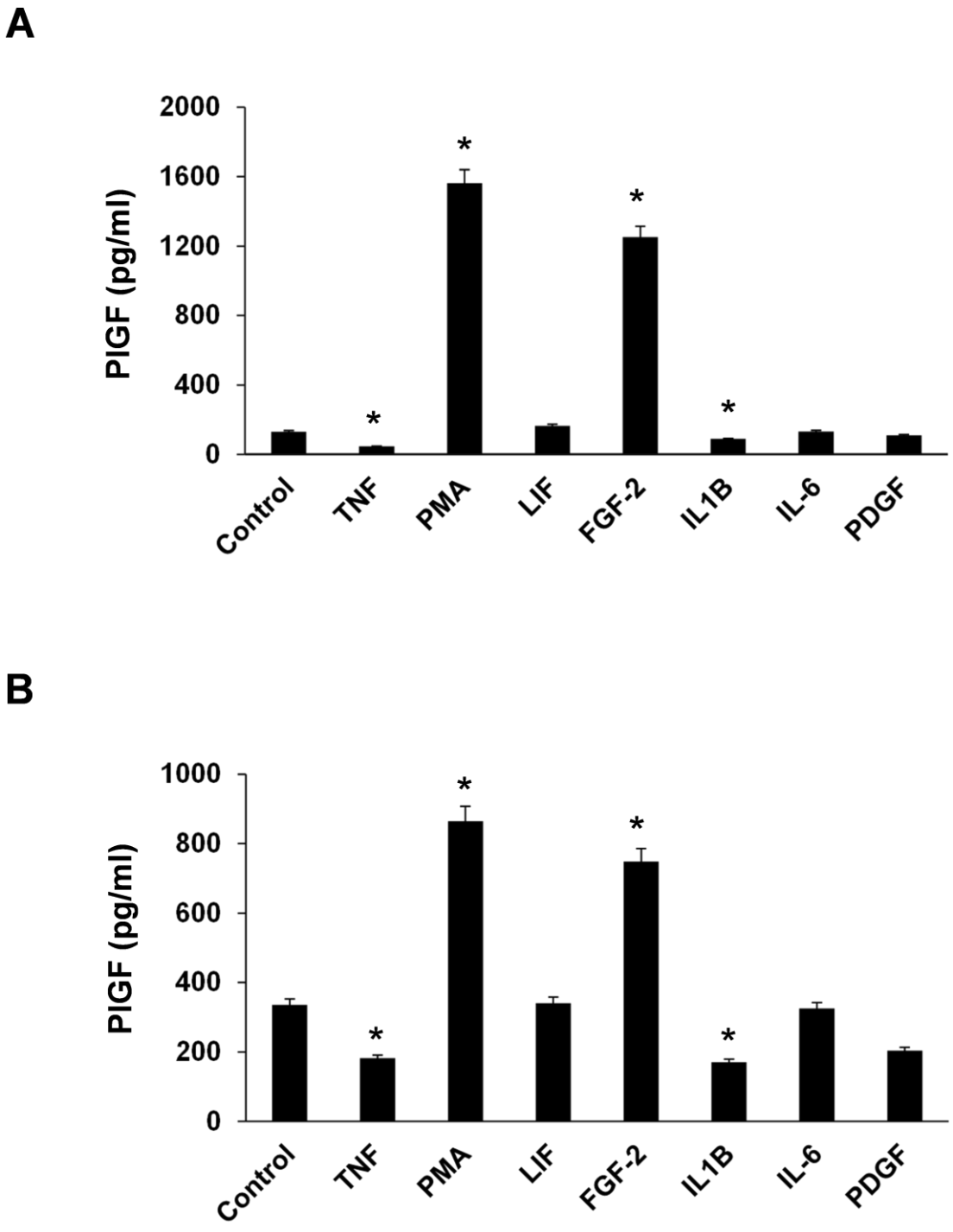


Figure 4.3 Factors regulating the release of PlGF from ECs*. ECs were seeded at a density of 1.5X105 per well in 24 well dishes, serum starved overnight in 5% FCS containing medium. Supernatants were collected 24 hours post stimulation with growth factor or cytokine. Cell supernatants were analysed for PlGF levels in HUVEC [A], HMEC-1 [B], by ELISA. Data are expressed in pg/ml of free and bound PlGF and are mean [± SEM] of three separate experiments performed in triplicates. All the growth factors or cytokines used at a concentration of 10 ng/ml and PMA at 100nM. *P<0.05 vs. Control.*

The levels of PlGF released by SMCs in the presence of TNF α, PMA or FGF-2 demonstrated a three-four-fold increase respectively when compared to the control.

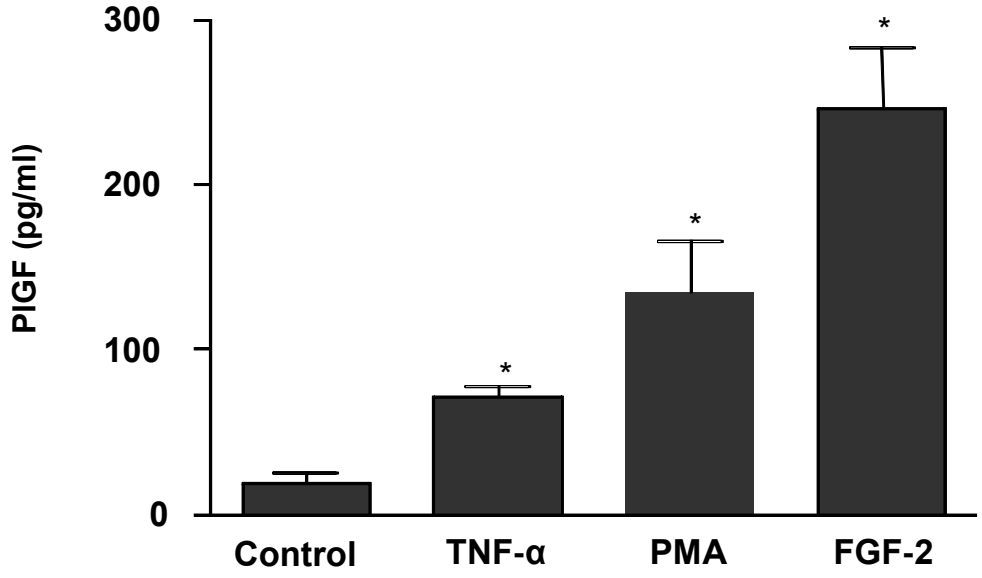


Figure 4.4 PlGF levels in stimulated SMCs. *Cells were seeded at a density of 1.5X105 per well in 24 well dishes, serum starved overnight in 5% FCS containing medium. Supernatants were collected 24 hours post stimulation with growth factor or cytokine. Cell supernatants were analysed for PlGF levels by ELISA. Data are expressed in pg/ml of free and bound PlGF and are mean [± SEM] of three independent experiments performed in triplicates. All the growth factors or cytokines used at a concentration of 10 ng/ml and PMA at 100nM. *P<0.05 vs. Control.*

4.2.3 Growth factors regulating PlGF release in ECs

PlGF, a homologue of VEGF (Maglione, Guerriero et al. 1991) has been demonstrated to play a synergistic role in pathologic angiogenesis, and is thought to ‘‘amplify’’ VEGF-driven angiogenesis and activation of vascular ECs (Autiero, Waltenberger et al. 2003). PlGF could therefore represent an additional and perhaps safer target [given its binding specificity for VEGFR-1], for the treatment of neovascular disorders. Microvascular cells exhibited higher levels of PlGF when screened for PlGF in our study, complying with previous reports (Yonekura, Sakurai et al. 1999, Zhao, Cai et al. 2004).

The effect of pro-angiogenic growth factors on PlGF release from ECs has been studied by measuring the levels of PlGF from HUVEC in response to treatments with a range of growth factors. VEGF is the most predominant growth factor regulating angiogenesis (Roy, Bhardwaj et al. 2006). Splice variant isoform VEGF-A_{165} induced PlGF release from ECs, in concurrence with earlier reports (Zhao, Cai et al. 2004), and seemed to be more potent than VEGF-A_{121}, which was reported to be remarkably similar to PlGF structurally (Iyer and Acharya 2002). VEGF-A_{165} seemed to be more potent agonist of PlGF, wherein, 10ng/ml of VEGF-A_{165} induced a three-fold increase in the levels of PlGF, compared to two-fold increase by VEGF-A_{121} at 50ng/ml (Figure 4.5A). VEGF–E, a highly related protein encoded by Orf virus, mediating angiogenesis preferentially via the VEGFR-2 signalling (Ogawa, Oku et al. 1998, Meyer, Clauss et al. 1999, Wise, Veikkola et al. 1999), seemed to have reduced the release of PlGF by two-fold compared to the control. Ubiquitous angiogenic factor FGF-2 induced an increase in the levels of PlGF by three-five-fold in a concentration dependent manner, similar to that of VEGF-A_{165}. Similarly, EGF, a known inducer of cancer risk, (Herbst and Shin 2002) inhibited PlGF levels (Figure 4.5B).

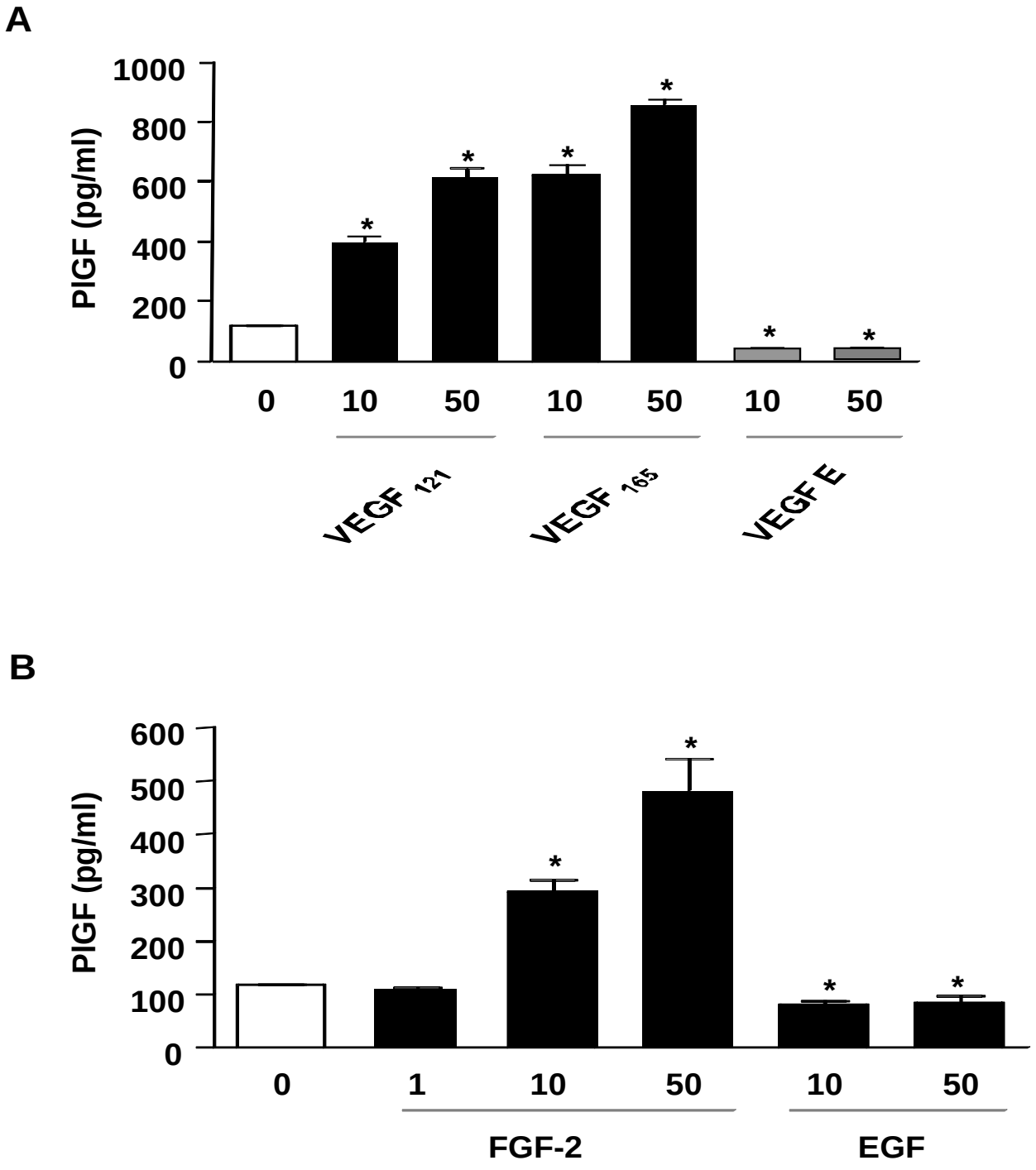


Figure 4.5 PlGF levels in response to growth factors. *HUVEC were seeded at a density of 1.5X105 per well in 24 well dishes, serum starved overnight in 5% FCS containing medium. Supernatants were collected 24 hours post stimulation with growth factors, VEGF, FGF-2 and EGF at specific concentrations mentioned in the figure. Cell supernatants were analysed for PlGF levels by ELISA. Data are expressed in pg/ml of free and bound PlGF and are mean [± SEM] of three independent experiments performed in duplicates. *P<0.05 vs. Control (0).*

4.3 Discussion

PlGF contributes to regulating the angiogenic switch, and its significant role in promoting aberrant angiogenesis in a variety of pathologies has gained acute attention in the past few years. However, little is known about the cell type specific expression of PlGF, and the mechanisms by which modulation of PlGF results to pathological angiogenesis are poorly understood. Studies over the years have demonstrated that several factors influence morphological and functional differences between the diverse ECs derived from microvascular endothelial beds. A variation in response to growth factors/cytokines pertains between these ECs, well documented by Aird (Aird 2007).

In this study we not only establish ECs to be the prime source of PlGF producers, but also the factors regulating PlGF levels in ECs. We also analysed VEGF levels in the same panels of ECs and cancer cell lines. ECs extracted from both macro and micro vasculature, such as umbilical vein, dermis, lung, adipose and aorta expressed significant high levels of PlGF mRNA and protein levels in contrast to a panel of cancer cell lines. This data conforms to previous studies reporting ECs to be the principal cell types expressing PlGF (Yonekura, Sakurai et al. 1999, Zhao, Cai et al. 2004). PlGF protein was below detectable levels in most of the cancer cell lines derived from breast, colon, lung and kidney carcinomas. Cancer cells have been reported to have high levels of VEGF (Tran, Master et al. 2002). VEGF levels in the same panels of both ECs and cancer cell lines were found to be diametrical to the levels of PlGF, contemporaneous to thyroid tumours, where an increase in the VEGF levels and a decrease in the PlGF levels were reported (Viglietto, Maglione et al. 1995). Non-ECs, such as fibroblasts [MRC-5], had negligible levels of PlGF secreted, whereas PlGF secretion by SMCs was significant. Increase in the PlGF levels secreted by SMCs was three-five fold lower than ECs and twofold higher than the cancer cell lines screened. In contrast, the levels

of VEGF released in both the cell types MRC-5 and SMCs was three to five-folds greater than the ECs. According to previous studies, PlGF was observed to stimulate SMCs synchronous to ECs during the induction of vessel growth in a more balanced manner than its homologues PDGF-BB or VEGF (Keyt, Nguyen et al. 1996), which opt to stimulate SMCs or ECs, respectively (Carmeliet and Conway 2001). This might be due to the common receptor, VEGFR-1 specific to PlGF, being present on both the cell types. In addition, our results show PlGF being produced by both the cell types; PlGF produced by ECs as well as SMCs indicates a possible existence of an autocrine regulation mechanism. However, further co-culture and in vivo studies are warranted. PlGF is expressed by migrating keratinocytes and ECs, acting in a paracrine and autocrine fashion on VEGFR-1-expressing endothelium (Failla, Odorisio et al. 2000). Lack of PlGF resulted in delayed wound closure, indicating that it is required for optimal skin repair (Carmeliet, Moons et al. 2001). Over expression of this growth factor accelerates wound closure in diabetic mice (Cianfarani, Zambruno et al. 2006).

Because both PlGF and VEGF-A share VEGFR-1 as their common receptor molecule (Korpelainen and Alitalo 1998, Nicosia 1998, Neufeld, Cohen et al. 1999), it is likely that the co expression of PlGF and VEGF-A might affect the binding of VEGF-A to its receptors and may thus modulate the cellular functions executed by VEGF-A or PlGF or VEGF/PlGF heterodimer. This would encompass the objective of the study to examine the role of PlGF or the factors modulating its production in ECs. Due to the evidence that HUVECs do not produce VEGF *in vitro,* we therefore committed to use HUVECs in this chapter to investigate the factors regulating PlGF secretion by ECs, which might dissect mechanisms underlying in a variety of pathologies. ECs treated exogenously with a range of cytokines and growth factors were assayed for PlGF by ELISA. VEGF, FGF-2, and PMA, a phorbol ester, pharmacological activator of PKC and tumour inducer (Furstenberger, Berry et al. 1981),

induced PlGF release in these cells, supporting previous reports with respect to VEGF induction of PlGF (Yao, Yang et al. 2005). We demonstrate that VEGF-A_{165} and VEGF-$A_{121,}$ secretory and circulating splice variants of VEGF-A (Ferrara and Davis-Smyth 1997), induced PlGF production in ECs. Nevertheless, VEGF-A_{165} seemed to be more potent agonist of PlGF than its contemporary homologue VEGF-$A_{121.}$ $VEGF_{165}$ has been reported to up regulate PlGF production from ECs (Yao, Yang et al. 2005), so does PlGF up regulate VEGF (Roy, Bhardwaj et al. 2005). Over expression of PlGF by adenovirus-mediated gene transfer increased both VEGF-A_{165} and VEGF-A_{121} protein in perivascular tissue, resulting in significantly enhanced angiogenesis (Roy, Bhardwaj et al. 2005). Recombinant treatment of VEGF-A_{121} in rat model of preeclampsia [PE] alleviated PE like symptoms including the plasma sVEGFR-1 levels, almost to the levels in the normal pregnant rat (Li, Zhang et al. 2007). Gene expression studies in pregnant rat model of PE demonstrated VEGF-A_{121} treatment reversed 125 of 268 sVEGFR-1 induced changes in gene expression of cluster genes related to angiogenesis, hypoxia, inflammation and coagulation (Gilbert, Verzwyvelt et al. 2010). The angiogenic cluster of genes reduced in this study did not show the status of gene encoding PlGF protein that is dramatically reduced in PE (Polliotti, Fry et al. 2003, Chaiworapongsa, Romero et al. 2009). Our study has shown that PlGF is the common factor induced by both of the ligands VEGF-A_{165} and VEGF-A_{121} which have been reported by above studies to be of therapeutic benefit in different pathologies. It might well be that this central key element PlGF is the potential target modulated, and maybe involved in the underlying mechanisms of these therapies. Taken together, these results indicate the existence of an autocrine mechanism involved in the release of these growth factors. VEGFR-2 specific ligand VEGF-E (Ogawa, Oku et al. 1998, Meyer, Clauss et al. 1999), which stimulates EC mitogenesis and permeability with a synonymous potency to that of VEGF-A (Ogawa, Oku et

al. 1998, Meyer, Clauss et al. 1999), inhibited the levels of PlGF secreted by ECs. This probably might have been due to the interference of the signalling pathways, such as MAPK and PI3K, involved in PlGF production, which also seems to be induced by VEGF-E. However, PlGF was shown to be superior than VEGF or VEGF-E in their contributions to collateral vessel formation (Pipp, Heil et al. 2003). This study also suggests that cooperation between the two receptors VEGFR-1 and VEGFR-2, either on cellular or signalling levels, does not seem to be required for their effects on inducing collateral growth *in vivo* (Pipp, Heil et al. 2003). Though VEGF-E is a viral homologue of VEGF family (Meyer, Clauss et al. 1999), the system would enable us to establish the VEGF family receptor specific driven angiogenesis, and a further insight into the potency of PlGF mediated angiogenesis, specifically via VEGFR-1 and the modulation of this system in the presence of VEGF-R2.

FGF-2 and EGF demonstrated a contrast effect on PlGF levels. FGF-2 induced the production of PlGF by ECs in a concentration-dependent manner. FGF-1 induced PlGF secretion with 20 percent lower potency than FGF-2 [data not shown]. FGF-2 also induced PlGF levels in SMCs about three-folds greater than control. FGF-2-mediated angiogenesis is dependent on VEGFR-1 activity in cultured HUVEC (Kanda, Miyata et al. 2004), which indicates the probability that FGF-2 might induce PlGF *in vivo* as well in order to be functional. Independent studies reported the beneficial effect of FGF-2 and PlGF in promoting angiogenesis, wound healing, restoring cardiac function (Harada, Grossman et al. 1994, Horrigan, Malycky et al. 1999, Iwama, Uemura et al. 2006, Kardami, Detillieux et al. 2007) and have also been named as key cytokines promoting inflammatory angiogenesis (Kanazawa, Tsunoda et al. 2001, Perelman, Selvaraj et al. 2003, Selvaraj, Giri et al. 2003(Perelman, 2003 #450, Zittermann and Issekutz 2006). We demonstrated that FGF-2 is a potent inducer of PlGF in ECs, and this may be the prime reason for the complementary

beneficial effect of FGF-2/PlGF in the above studies. The correlation between the two growth factors has been studied in detail in subsequent results chapters. This includes the underlying signalling mechanism for FGF-2-mediated PlGF release is studied in detail in succeeding chapter.

EGF, a known inducer of cancer and angiogenesis (Ongusaha, Kwak et al. 2004), decreased the levels of PlGF. However, this inhibition was not concentration-dependent.

GM-CSF, IL-1β and TNF α had an opposite effect, inhibiting the levels of PlGF twofold. Angiogenic and anti-angiogenic factors represent the balance and function of vascular endothelium. Deviation from this equilibrium may lead to chronic inflammatory diseases, such as preeclampsia [PE] (Sharma, Satyam et al. 2007), rheumatoid arthritis (Folkman 1995), psoriasis (Braverman and Sibley 1982) or Crohn's disease (Kanazawa, Tsunoda et al. 2001). Hollborn et al study report on PlGF secretion by human epithelial retinal cells [RPE] had a contrast result (Hollborn, Tenckhoff et al. 2006), when compared to our study. VEGF, PDGF and pro-inflammatory cytokines such as TNF α, IL-1β had no effect on PlGF release from RPEs. However, FGF-2 seemed to have decreased the levels of PlGF by about 50%, which was not acknowledged in the study. This might have been due to the low levels of PlGF expressed in this specific epithelial cell type. The secreted concentration could not be determined as the results being expressed in percentage of control (Hollborn, Tenckhoff et al. 2006). It clearly indicates the cell type specific PlGF levels and their varying effect in the presence of growth factor and cytokines on PlGF production. Inflammatory cytokines are peptide mediators of EC activation as well as dysfunction (Sharma, Satyam et al. 2007). TNF-α is a potent Th1-type pro-inflammatory cytokine and has been reported to cause direct damage to ECs, increase EC permeability, up-regulate endothelial adhesion molecules [ICAM-1, VCAM-1, E-Selectin] and promote vasoconstriction, in the pathologies (Hunt,

Chen et al. 1996). These results hint the effect of Th1 type cytokines, such as TNFα, on PlGF levels, which has been the centre of the succeeding results chapter in this study.

Chapter 5 PlGF Regulation by FGF-2 and Signalling systems mediating FGF-2-driven PlGF release in ECs

5.1 Introduction

Recent studies have stated PlGF to have superior angiogenic activity than VEGF or FGF-2 induced angiogenesis, with transiently improved neovascularisation and overall less successful outcomes (Wu, Claus et al. 2016) (Table 1.3). FGF-2 is ubiquitous, and therefore, thought to have greater angiogenic efficacy and safety than other angiogenic factors, such as VEGF, PDGF (Cao, Brakenhielm et al. 2003) (Table 1.4). Relatively high levels of PlGF mRNA and protein (Figures 4.1 – 4.2) are produced by ECs, suggesting a potential autocrine role for PlGF in pathological angiogenesis. FGF-2 increased PlGF release in HUVEC and HMEC-1 (Figure 4.3). Elevated VEGF levels are positively correlated with cancer progression (Savaskan 2013). Orthotopic mouse model studies reported that PlGF overexpression inhibited tumour growth, angiogenesis and metastasis by depleting VEGF homodimers (Xu, Cochran et al. 2006). Together, these reports lay emphasis on the beneficial and safe aspects of PlGF and FGF-2 for potential therapy.

Binding of FGF-2 to FGFRs activates cellular responses by triggering multiple downstream signalling events (Klint and Claesson-Welsh 1999). FGF-2 administration prevents ischemia or reperfusion injury in animal models via activation of the protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) signalling cascades (Padua, Sethi et al. 1995, Padua, Merle et al. 1998, House, Melhorn et al. 2007). These include activation of the Ras-dependent signalling pathway via the recruitment of Src proteins, and subsequent induction of the Ras-Raf cascade and activation of the Ras-independent pathway, by PI3K and/or PKC/PLD (Ahmed, Plevin et al. 1994, Alavi, Hood et al. 2003, Langford, Hurford et al. 2005).

PlGF was found to be the most abundant of the other VEGF family members expressed in human dermal microvascular cells, at 100-fold greater than VEGF, which was the least abundant (Yonekura, Sakurai et al. 1999). Well characterised endothelial cell line HMEC-1 (Ades, Candal et al. 1992) grow in the absence of serum, compared to the primary vascular ECs which require 20-30 % serum supplemented medium (Ades, Candal et al. 1992). We observed increasing endothelial PlGF levels in response to the increasing serum concentrations (data not shown). Several factors endorsed the use of HMEC-1 for signalling studies in this chapter.

1. Confirmation of crucial findings with HUVEC for instance; Endothelial PlGF levels having similar response to FGF-2 in HUVEC as well as HMEC-1 (Figure 4.3).
2. Further investigation and experimental details of FGF-2-induced PlGF followed transcriptional regulation in HMEC-1 similar to findings in the next result chapter (Figure 6.1 – 6.2).
3. Robust nature of the HMEC-1 to grow in growth medium supplemented with low serum (0.5%).
4. Cells being easily available and could be used at higher passage numbers.
5. Several and recent studies used HMEC-1 for endothelial cell signalling as well as functional studies (Weinkopff and Lammie 2011, Asteriti, Daniele et al. 2012, Pontillo, Espanol et al. 2015).

Hence HMEC-1 was the chosen endothelial cell line to study, in an efficient way, the signalling pathway(s) governing FGF-2-induced endothelial PlGF. Pharmacological inhibitors, adenoviral-mediated overexpression systems and gene-silencing siRNA technology were used to delineate the signalling pathway(s) governing the release of PlGF in ECs. This

study demonstrates that under basal conditions, endothelial PlGF release involves Ras/Raf/MEK, PKC and PI3K pathways, while FGF-2-mediated PlGF release specifically requires MEK and PKC activation. HMEC-1 was used to study the signalling mechanism in this Chapter, primarily due to their robust nature, easy availability and more importantly due to the confirmation of crucial findings in FGF-2-induced PlGF results in HMEC-1 confirmed in HUVEC.

5.2 Results

5.2.1 FGF-2-mediated PlGF release is concentration-dependent

To investigate whether FGF-2-induced PlGF release was concentration-dependent, HMEC-1 cell lines were treated with increasing concentrations of FGF-2 for 24 hours. A concentration-dependent increase in the levels of PlGF release in cell supernatants was observed in response to the increasing concentrations of FGF-2. PlGF secretion reached statistical significance after addition of 1, 10 or 50 ng/ml of FGF-2 compared to the untreated cells. Treating HMEC-1 with 10 ng/ml FGF-2 caused a significant (two- to three-fold) increase in PlGF levels in HMEC-1 (Fig 5.1) and was therefore chosen concentration for subsequent experiments.

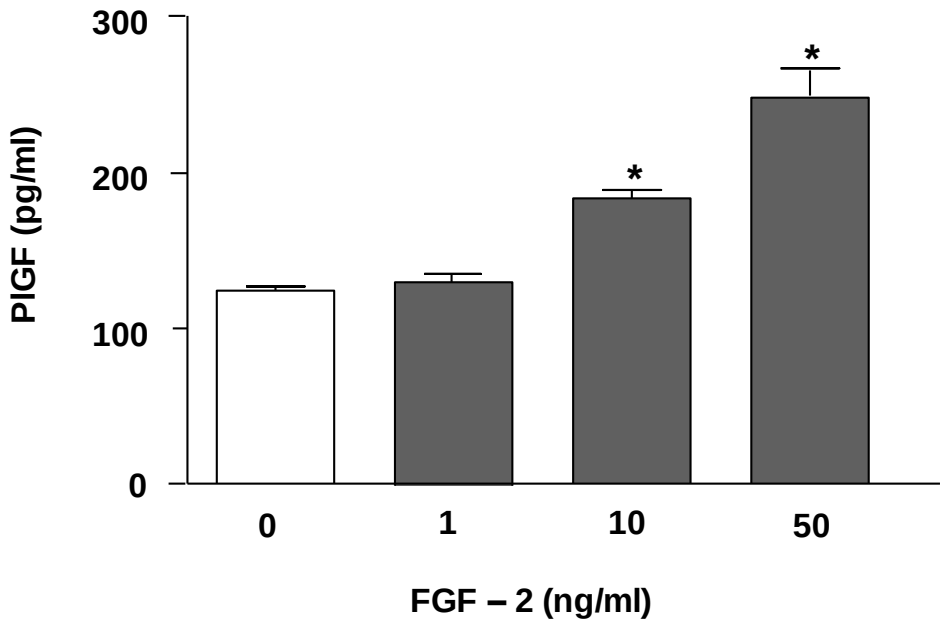


Figure 5.1 FGF-2 up-regulates PlGF release in a concentration-dependent manner. *HMEC-1 were cultured in serum-reduced RPMI medium containing 0.5% FCS and incubated overnight. ECs were stimulated with concentrations of FGF-2 for 24 hours. The conditioned media was collected and assayed for PlGF by ELISA. The data represent the mean (± SEM) of at least 4 independent experiments, performed in duplicate. * $p<0.05$ vs. Control.*

5.2.2 FGF-2 induces PlGF expression in ECs in a time-dependent manner

To investigate the kinetics of FGF-2-induced up-regulation of PlGF protein, HMEC-1 cells were treated with FGF-2, and PlGF levels were measured at various time points. Differences in the levels of PlGF release between FGF-2 treated and control cells became apparent after 8 hours, and was two-fold greater in FGF-2 stimulated cells at 16 hours, reaching a plateau at 24 hours (Figure 5.2A). Optimal time for subsequent experiments was therefore selected as 24 hours, unless specified. Real-time PCR analysis on RNA from HMEC-1 treated with FGF-2 for various time periods revealed that an increase in PlGF mRNA expression preceded the rise in PlGF protein secretion (Figure 5.2B). FGF-2-induced a four-fold increase in PlGF mRNA levels at 8 hours compared to its control, and returned to baseline levels by 24 hours. Cells were incubated overnight in low serum medium, followed by fresh medium with or without FGF-2 before collecting RNA samples at different time points for real-time PCR. This could explain the sudden increase in the mRNA levels at 8 hours. However, it is noteworthy that PlGF protein stimulation by FGF-2 is significant at 16 hours. Therefore, it appeared that FGF-2 may regulate PlGF expression at a transcriptional level. The average doubling time for both HUVEC and HMEC-1 is between 24-30 hours. For subsequent experiments 24 hours' time point is chosen unless otherwise specified.

Collectively, these results show that FGF-2-induced PlGF release is concentration-dependent through transcriptional activation of PlGF gene and *de novo* protein synthesis. Confirming these facts facilitated in proceeding to study in detail the signalling molecules involved in FGF-2-mediated PlGF release in ECs.

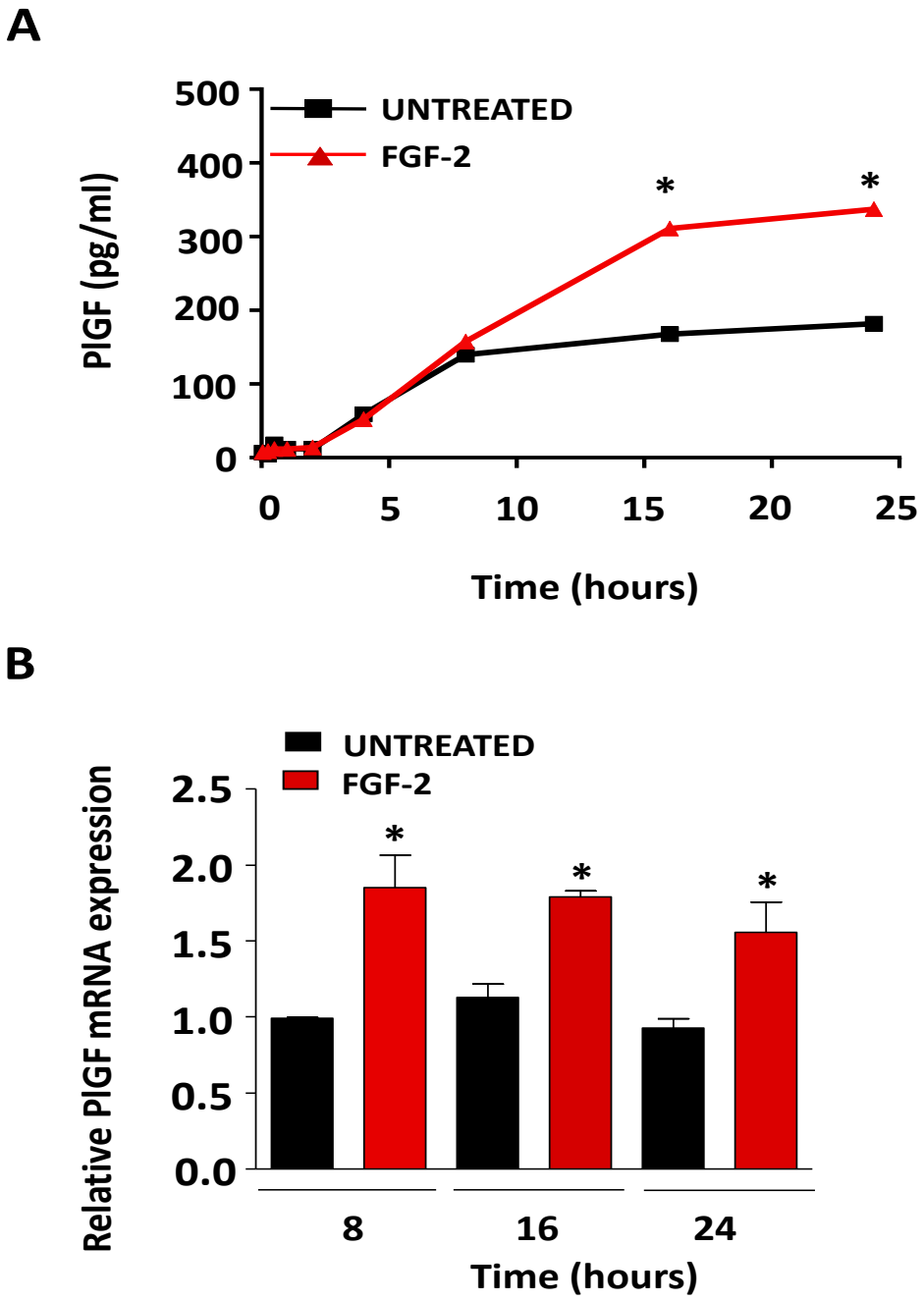


Figure 5.2 Kinetics of FGF-2-induced release of PlGF in cultured HMEC-1. *[A] Time-dependent PlGF protein release from HMEC-1 in response to FGF-2 [10 ng/ml] stimulation. Cell supernatants from conditioned medium were collected at indicated time points and PlGF levels measured by ELISA. [B] FGF-2-induced PlGF mRNA expression in a time-dependent manner. HMEC-1 were stimulated with FGF-2 (10 ng/ml) and mRNA subjected to quantitative real-time PCR for PlGF. PlGF mRNA values are normalized to β-actin gene expression and presented as mean (± SEM). Data are mean [±SEM] of at least three separate experiments performed in duplicate. * $p<0.05$ vs Untreated.*

5.2.3 Role of PKC in FGF-2-induced PlGF release from ECs

PKC is known to be involved in mediating FGF-2-induced EC function (Kent, Mii et al. 1995). We therefore investigated the possibility that FGF-2-induced PlGF production was regulated by PKC. We used the pharmacological PKC inhibitors Ro32-0432 and GO6976 to determine the role of PKC in FGF-2-mediated PlGF release. FGF-2 induced PlGF protein release was completely blocked to the basal level when HMEC-1 were treated with Ro32-0432 [Figure 5.3A] and GO6976 [Figure 5.3B] for 24 hours. A similar effect was elicited by these PKC inhibitors on PMA, an activator of PKC (Furstenberger, Berry et al. 1981), that induced PlGF protein levels in ECs (Appendix II – PMA concentration graph). These data indicate the key role of PKC in PlGF production in ECs.

5.2.3.1 Role of PKC isoforms in PlGF expression in ECs

The PKC family consists of several distinct isozymes, each of which have specific structural and biochemical properties and regulatory mechanisms (Dekker and Parker 1994). Cultured ECs (HUVEC) express PKC isozymes α, β, δ, ε, θ and ζ (Haller, Ziegler et al. 1996). PKC is a central regulator of angiogenesis (Tsopanoglou, Pipili-Synetos et al. 1993). We investigated the role of PKC in endothelial PlGF release by stimulating HMEC-1 with PKC activators. Thymeleatoxin [Thx], which is a selective activator of classical PKC isoforms [cPKC; α, β, and γ] (Ryves, Evans et al. 1991), Ingenol 3, 20-dibenzoate [IDB], which is a specific activator of novel PKC isoforms [nPKC; δ, ε, η, and θ], (Asada, Zhao et al. 1998) and á-APP modulator [á-APP] which is a selective activator of PKCα (Kozikowski, Nowak et al. 2003) were used for these studies. After 24-hours incubation in serum deprived conditions, PlGF levels were measured by ELISA in cell-conditioned medium. PKC activators, Thx and IDB

increased PlGF release by two- to three-fold (Figure 5.1C) compared to control. The á-APP modulator [á-APP] had the least effect on PlGF release.

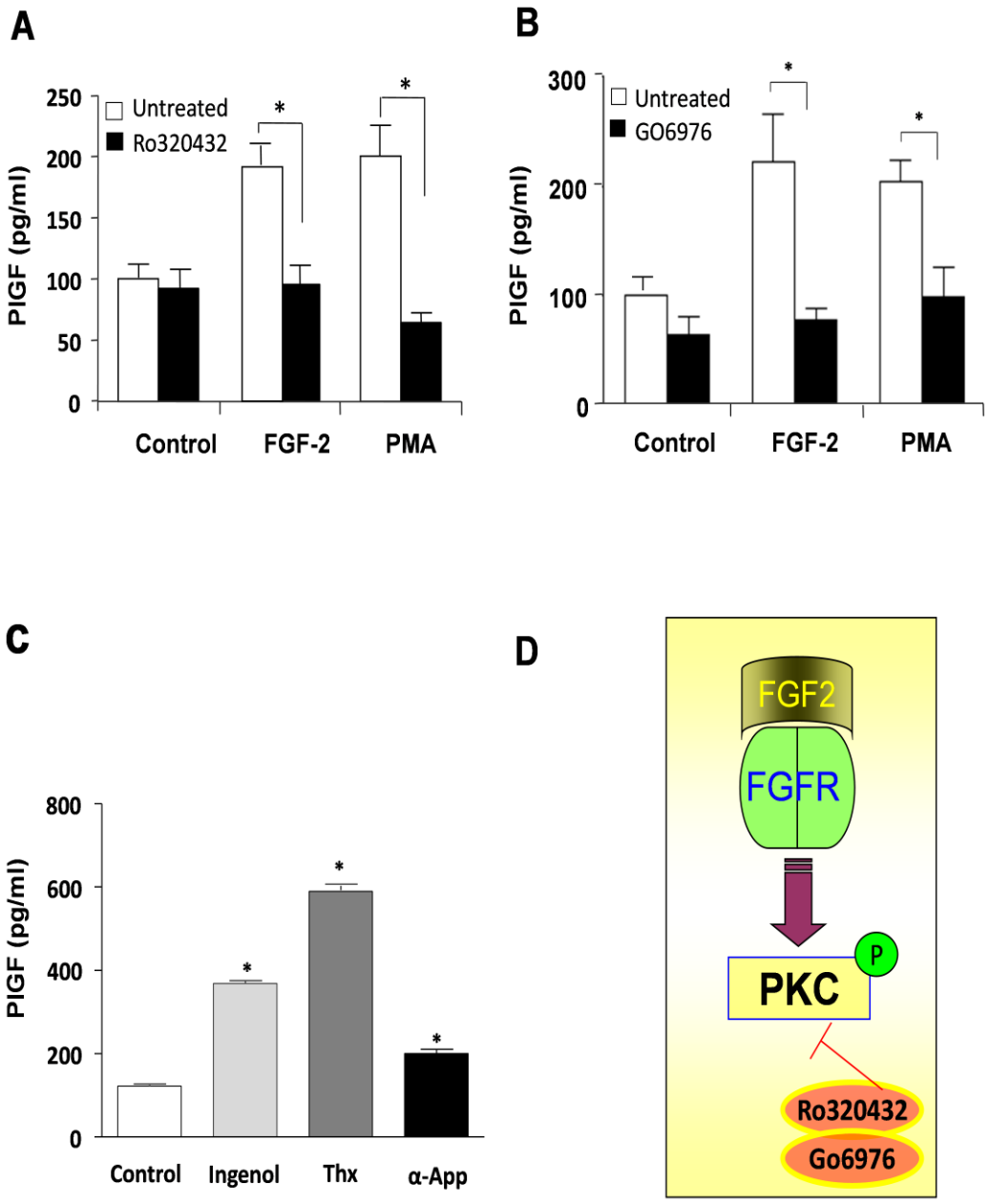


Figure 5.3 PKC is involved in FGF-2 mediated PlGF release in HMEC-1. *HMEC-1 were cultured in serum-reduced medium overnight and incubated with PKC inhibitors (A) Ro320432 (1 µM), (B) GO6976 (1 µM), 45 minutes prior to addition of stimulants FGF-2 (10 ng/ml) or PMA (100 nM). Cell supernatants were analysed for PlGF by ELISA.(C) PKC activators Ingenol 3, 20-dibenzoate (IDB) (1 µM), Thymeleatoxin (Thx; 10 µM) and α-APP (10µM) induce PlGF release in ECs. HMEC-1 were treated with PKC activators; for 24 hours and PlGF measured in cell supernatants. (D) Schematic illustration of PKC inhibition on FGF-2 signal transduction. Data are expressed as percentage release of PlGF measurements of 4 independent experiments performed in duplicate. *P<0.05 compared to the control.*

5.2.3 PKC isoforms involved in FGF-2-mediated PlGF release

To identify the PKC isoform[s] involved in FGF-2 stimulated PlGF release, HMEC-1 were infected with adenoviruses over-expressing dominant-negative PKC isoforms prior to stimulation with FGF-2 or PMA. Over-expression of dominant-negative isoforms α, δ and ε significantly decreased PlGF levels in the presence of FGF-2 or PMA (Figure 5.4). The decrease in PlGF levels was significantly greater for dominant-negative PKCα treated samples compared with dominant-negative PKCδ or PKCε treated samples.

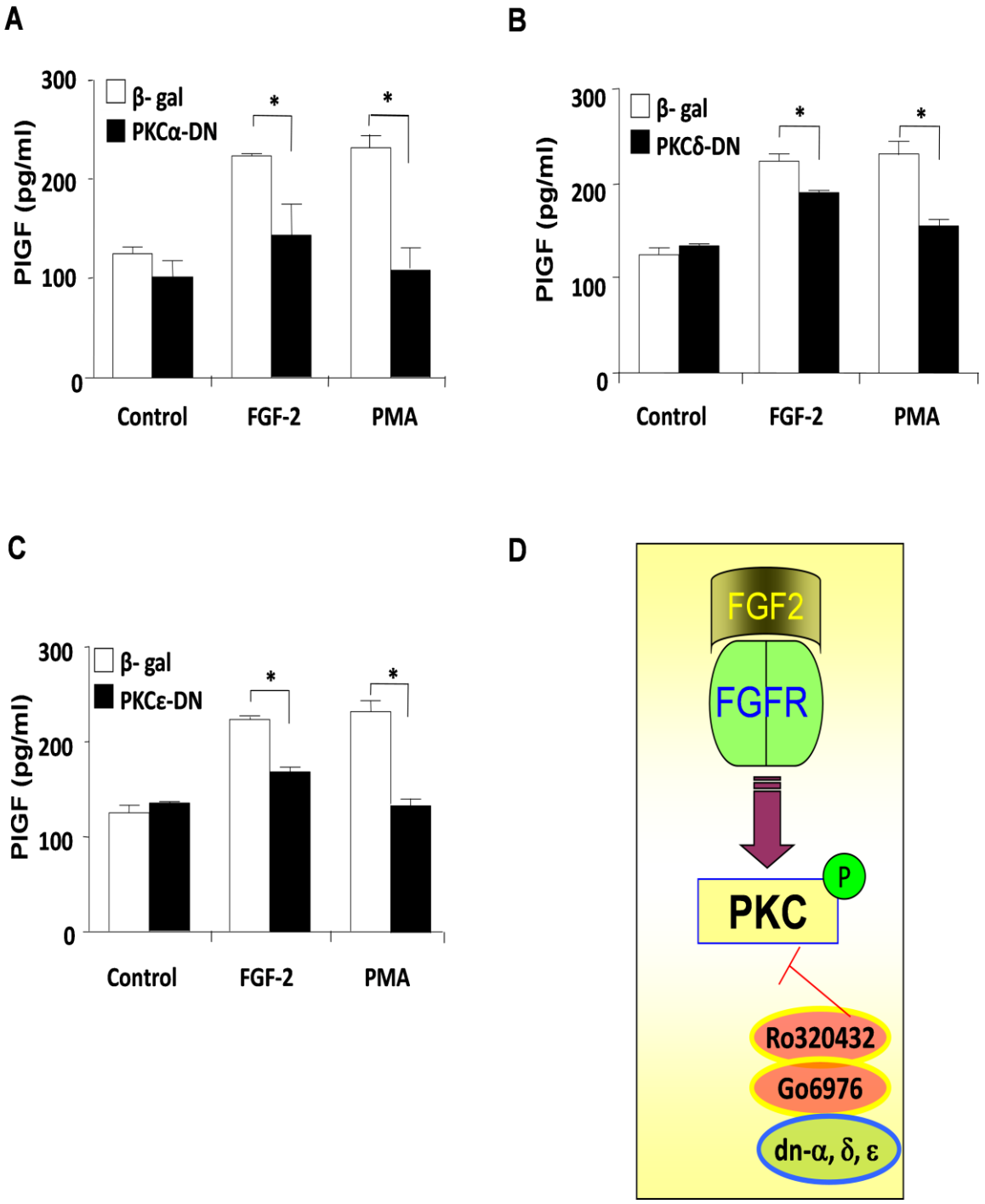


Figure 5.4 PKC isoforms involved in FGF-2 mediated PlGF release. *HMEC-1 were infected with expressing adenoviruses β -galactosidase [β-Gal] or recombinant adenoviruses encoding dominant-negative PKC isozymes of [A] PKC α, [B] PKCδ or [C] PKCε for 24 hours prior to incubation with 10 ng/ml FGF-2 or 100 mM PMA in basal medium containing 0.5% FBS. PlGF was measured in cell supernatants by ELISA.(D)Pictorial depiction of PKC inhibitors effecting FGF-2 signal transduction. Results are the means [±SEM bars] of at least 3 separate experiments [n=6] performed in duplicate. *P<0.05 compared to the control.*

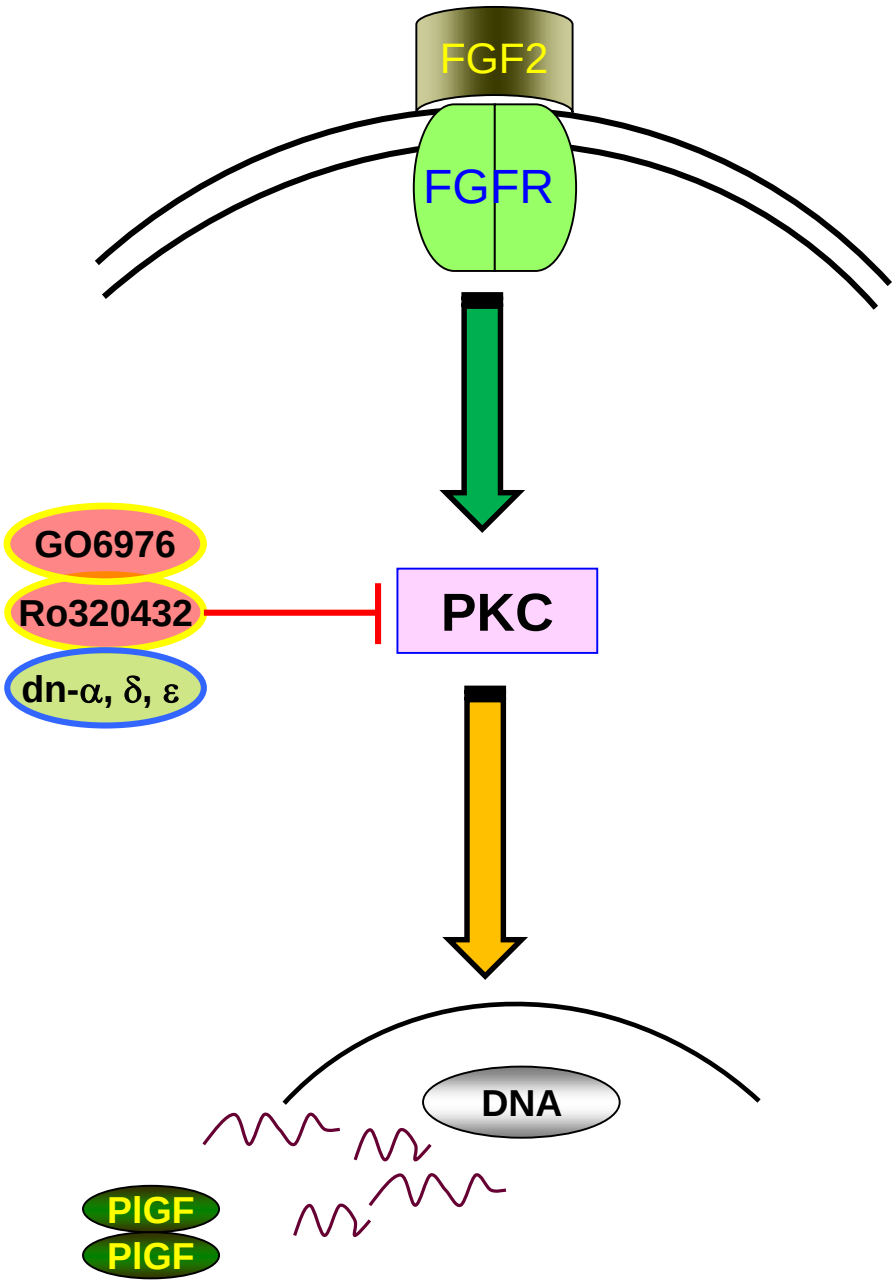


Figure 5.5 PKC isoforms involved in FGF-2 mediated PlGF release. *PKC inhibitors effecting downstream signalling of FGF-2 thereby obstructing FGF-2-induced PlGF procduction.*

5.2.4 FGF-2- stimulated PlGF secretion from ECs requires PLD activity.

Activation of PLCγ or PLD leads to the production of secondary messengers which phosphorylate PKC (Nishizuka 1995, Kook and Exton 2005). FGF-2 stimulates PLD signalling via PKC activation in ECs, independent of PLCγ (Ahmed, Plevin et al. 1994). We therefore investigated the role of these signalling molecules in FGF-2-mediated PlGF release from ECs. HMEC-1 were treated with U71322, a phosphatidylinositol-specific PLCγ (PI-PLCγ) inhibitor or the PLD inhibitor, 1-Butanol or its isomer control, 2-Butanol, in the presence of FGF-2. PlGF levels in the control and FGF-2 treated samples appear to be secreted differentially following PI-PLCγ inhibition, compared to the treatment with 1-butanol. PI-PLCγ inhibitor partially suppressed FGF-2-dependent PlGF protein levels (Figure 5.6A), in contrast to the PlGF protein levels in the absence of FGF-2. However, this suppression in protein concentration was very small, suggesting that PI-PLCγ is not involved in FGF-2-mediated PlGF release from ECs. Basal and FGF-2-induced PlGF levels were radically reduced when PLD was blocked using 1-Butanol (0.4 % v/v) (Figure 5.6B), whereas treatment with 2-Butanol did not affect PlGF release. These data clearly demonstrate the loss of basal production of PlGF as well as FGF-2 stimulated PlGF secretion following inhibition of PLD, in contrast to the small reduction in FGF-induced production following to PLCγ inhibition.

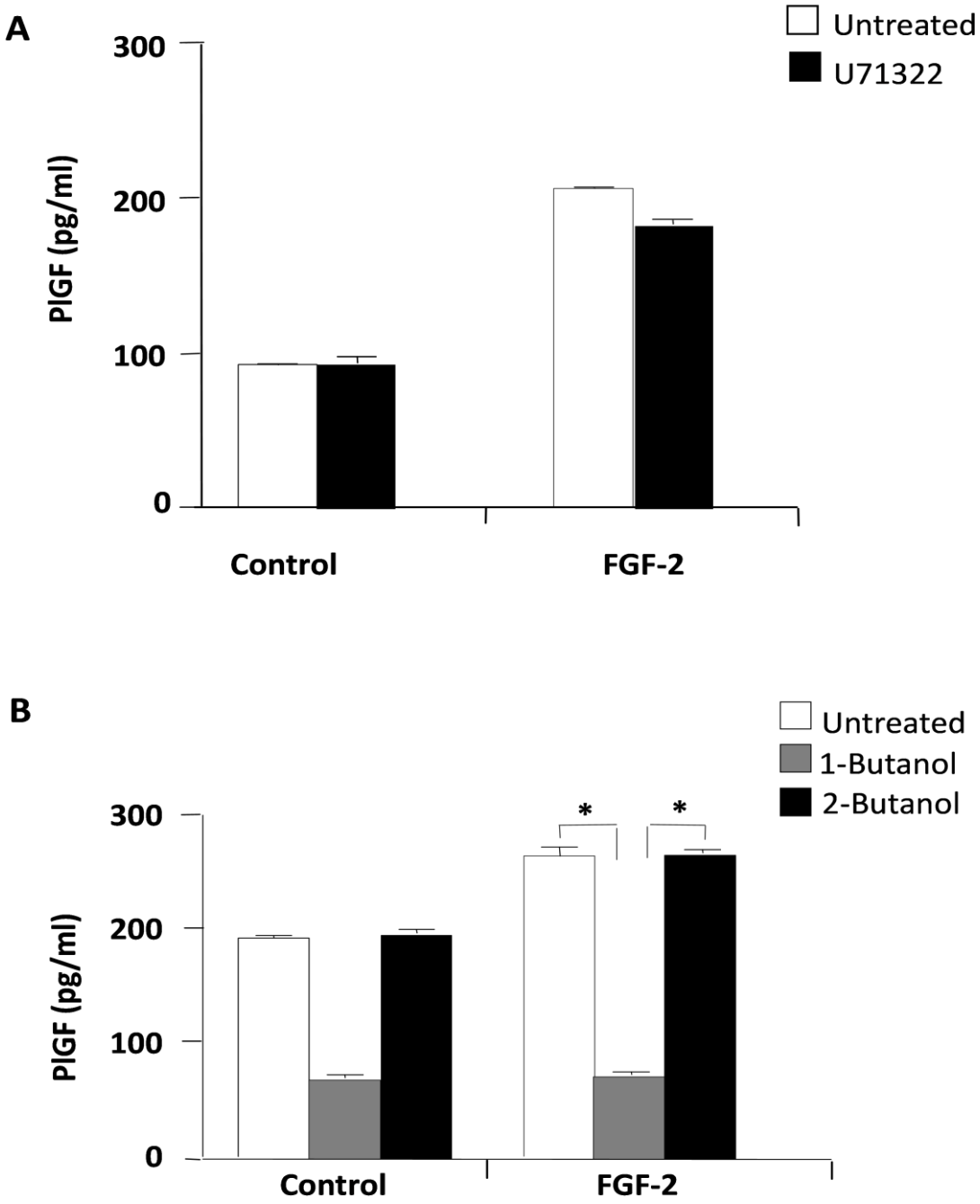


Figure 5.6 Role of PLD in PlGF release from ECs. *HMEC-1 were cultured in serum-reduced medium overnight and incubated with PI-PLCγ inhibitor (A) U71322 (0.1μM) or (B) 1-butanol (0.04% v/v) or its isomer control, 2-butanol (0.04% v/v), for 45 minutes prior to addition of FGF-2 (10 ng/ml). Cell supernatants collected following 24 hour incubation and analysed for PlGF by ELISA. Data are expressed as means ± SEM of measurements of 3 independent experiments performed in duplicate. *p<0.01 vs. control.*

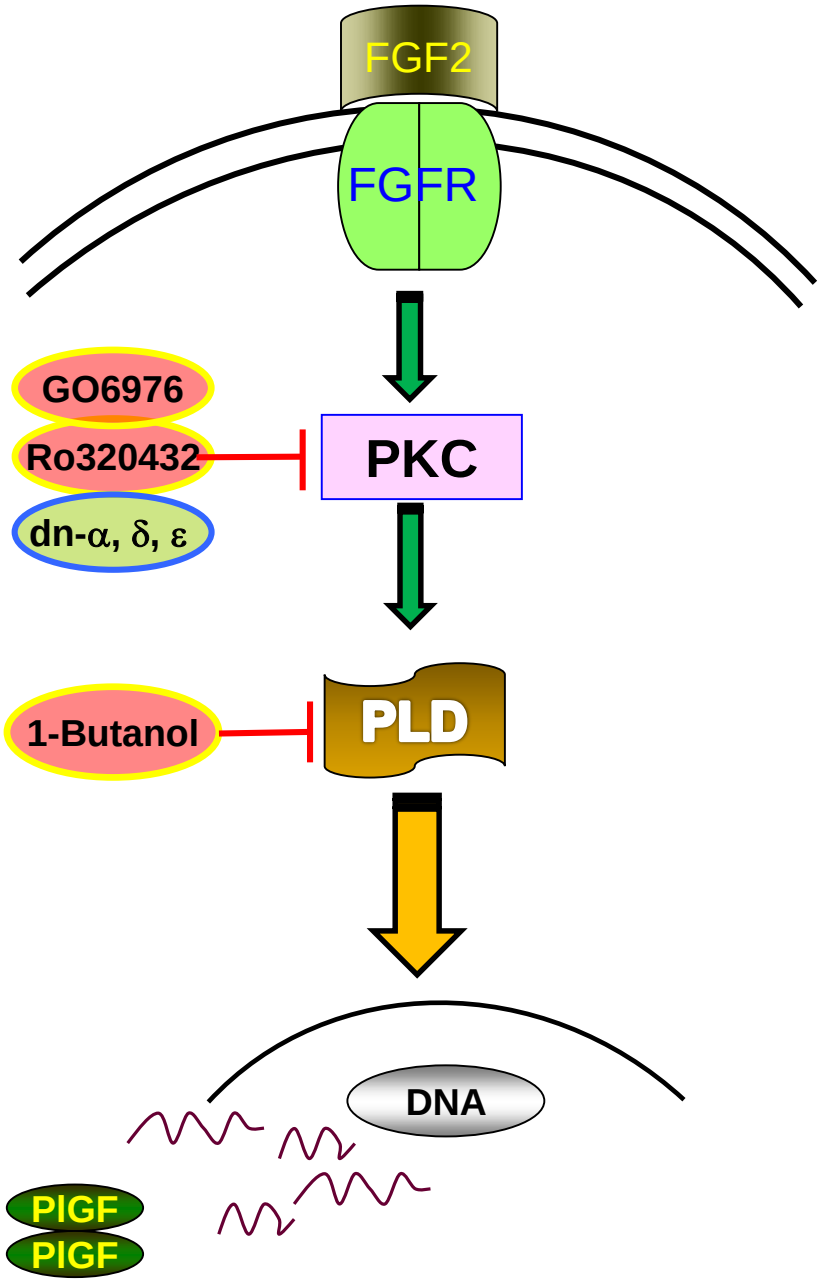


Figure 5.7 Diagrammatic illustration of FGF-2-mediated PlGF is PLD dependent.

5.2.5 FGF-2 stimulates the release of PlGF via the Ras-Raf-MEK Pathway

PKC isoforms activate Raf-1 (Cai, Smola et al. 1997, Schonwasser, Marais et al. 1998) and FGF-2 is reported to stimulate ERK1/2 MAPK phosphorylation in ECs (Yang, Wang et al. 2008). We assessed the involvement of the Ras-Raf-MEK pathway (Figure 5.4A) on PlGF expression by examining ERK1/2, a down-stream effector of MEK1/2 (Bikfalvi, Klein et al. 1997). Both MEK1/2 and ERK1/2 belong to the MAPK pathway that govern the processes of proliferation, differentiation and cell survival (Kolch 2000). Moreover, p38 MAPK negatively regulates endothelial cell differentiation in FGF-2 mediated angiogenesis (Tanaka, Abe et al. 1999, Matsumoto, Turesson et al. 2002). We used the MEK inhibitor, PD98059 to examine the involvement of MAP kinase in FGF-2-induced PlGF release, as this inhibitor efficiently suppresses the activation of p42/p44 MAPKs. Inhibition of MEK attenuated FGF-2-mediated PlGF release by ECs to basal levels [Figure 5.8A]. PMA, an activator of ERK1/2 signalling pathway (Verin, Liu et al. 2000), also significantly decreased PlGF levels in the presence of the MEK inhibitor PD98059. Blockade of p38 MAPK using SB203580 had no effect on FGF-2-mediated PlGF release (Figure 5.8B). These results indicate that MEK plays a significant role in FGF-2-mediated PlGF release, though p38 MAPK does not.

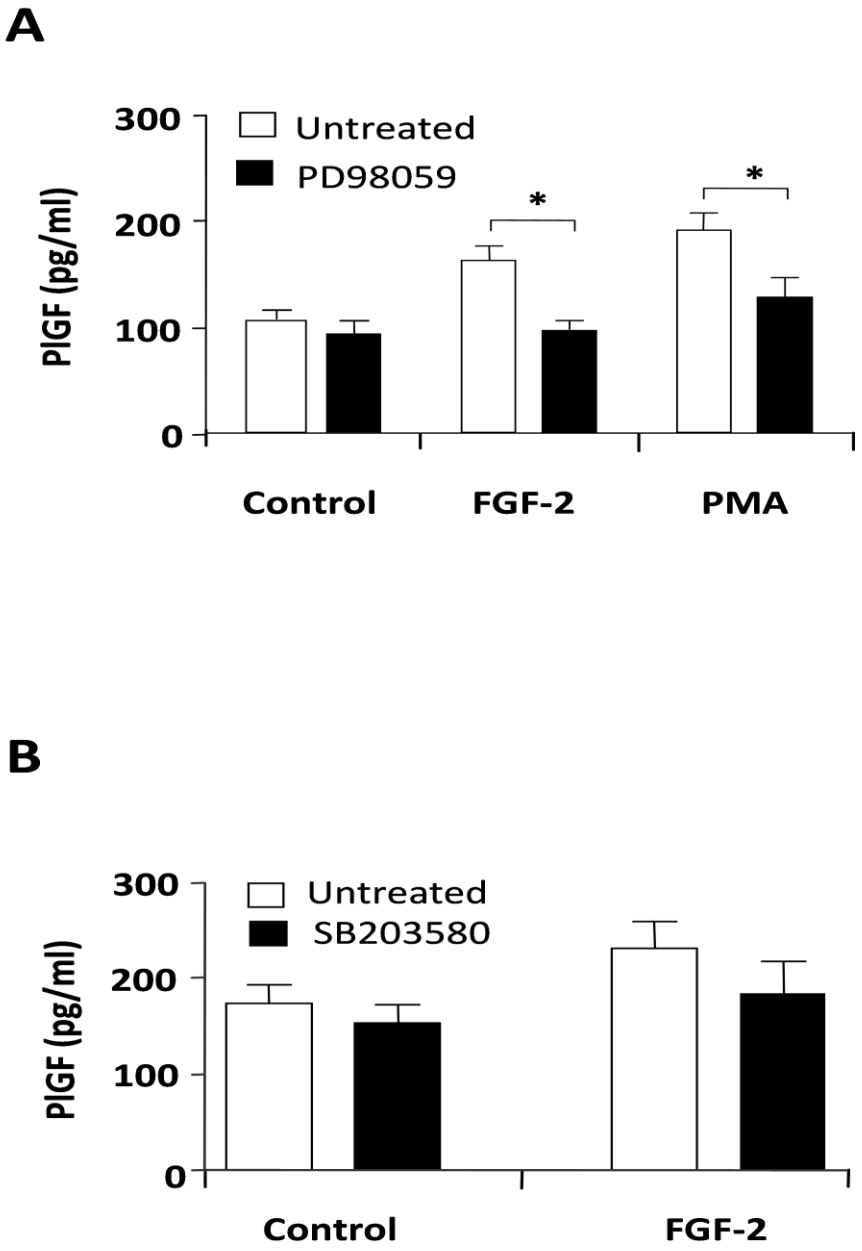


Figure 5.8 Role of Ras/MAPK signalling in FGF-2-induced PlGF release. *HMEC-1 were cultured in serum-reduced medium overnight and pre-incubated for 45 minutes with pharmacological inhibitors (A) PD98059 (20 µM) or (B) SB203580 (20 µM) prior to the treatments with FGF-2 (10 ng/ml) or PMA (100 nM). Cell supernatants collected following 24 hour incubation and were analysed for PlGF by ELISA. Data are expressed as means ± SEM of measurements of 3 or 4 independent experiments performed in duplicates. *p<0.01 vs control.*

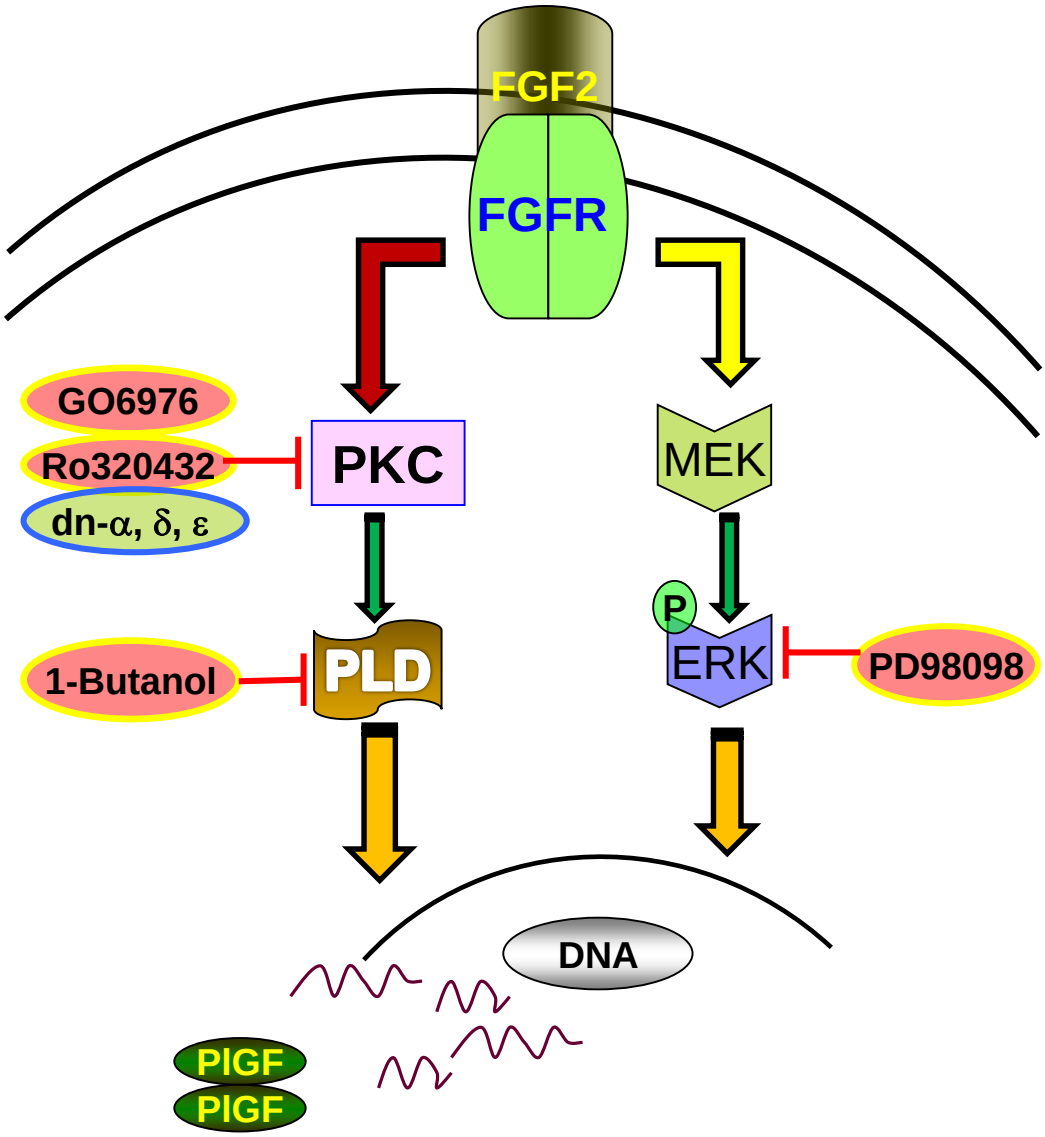


Figure 5.9 Diagrammatic representation of Ras-Raf-MAPK signalling pathway. Effect of MEK inhibitor on FGF-2 treated PlGF release.

Ras is a highly conserved protein which serves as a convergence point for multiple signalling pathways. Ras is up-stream of the MAPK linear cascade and is involved in the phosphorylation of ERK (White, Nicolette et al. 1995, White, Vale et al. 1996). Ras has been reported to be involved in FGF-2-induced endothelial cell differentiation (Klint, Kanda et al. 1999)**.** To study the role of Ras in FGF-2-mediated PlGF release, cells were infected with an adenovirus expressing a dominant-negative mutant of Ras [AdRas^{DN}], PlGF levels were measured in the cell supernatants. Over-expression of Ras^{DN} in HMEC-1 inhibited PlGF release by 80% in both FGF-2 stimulated and untreated cells, indicating that Ras plays a pivotal role in endothelial cell PlGF release (Figure 5.10A). Furthermore, dominant-negative over expression of Ras^{DN} abrogated ERK1/2 phosphorylation in both the untreated and FGF-2-treated protein samples (Figure 5.10B). This confirms that Ras activity is required for PlGF release via the downstream activation of ERK1/2. However, partial inhibition of ERK1/2 activation subsequent to Ras blockade in the presence of the PKC activator PMA was observed. This indicates the involvement of PKC signalling pathway independent of ERK1/2 in FGF-2 induced PlGF release from ECs.

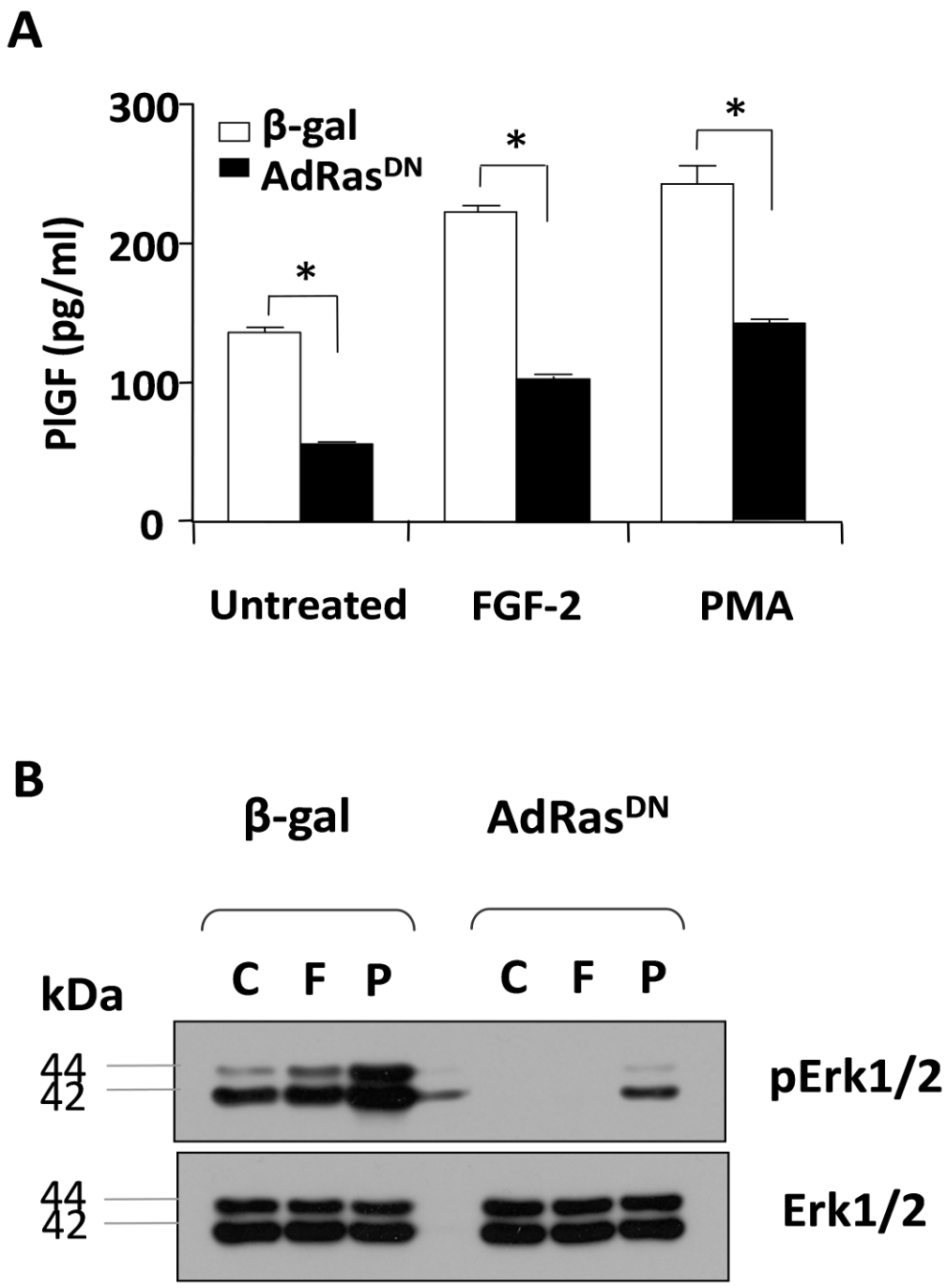


Figure 5.10 Ras involved in FGF-mediated PlGF release. *(A) HMEC-1 were infected with adenovirus encoding dominant-negative Ras (AdRasDN) or β -galactosidase (β-Gal) control for 24 hours. Cells were treated with FGF-2 (10 ng/ml) or PMA (100nM) for 24 hours in basal medium containing 0.5% FBS. PlGF was measured in cell supernatants by ELISA. (B) Representative Western blot showing the effect of RasDN on ERK1/2 phosphorylation following FGF-2 or PMA stimulation. HMEC-1 infected with adenovirus encoding dominant-negative Ras (AdRasDN) or β -galactosidase (β-Gal) control for 24 hours were stimulated with FGF-2 or PMA for 15 minutes. 25µg of cell protein per sample was separated by 10% SDS-PAGE. Activation of ERK1/2 was determined by Western-blot analysis using antibodies*

*that recognized phosphorylated ERK1/2 (pERK or p42/44). C – Control, F – FGF-2 (10 ng/ml), P– PMA (100 nM). Data are expressed as means ± SEM of measurements of 3 or 4 independent experiments performed in duplicates. ** P<0.01 vs control.*

5.2.6 Src Kinase activity is critical for PlGF release from ECs

The preceding results confirmed that Ras-Raf-MAPK and PKC were critical for FGF-2-mediated PlGF release from ECs. Src kinase has previously been reported to be involved in FGF-2/FGFR signalling (Klint, Kanda et al. 1999). Cells were treated with PP2, a potent inhibitor of the Src family of tyrosine kinases (Hanke, Gardner et al. 1996). Inhibition of Src kinase activity abrogated FGF-2-induced PlGF release in a concentration-dependent manner (Figure 5.11A). To elucidate the potential involvement of Src kinase on downstream signalling events, we assessed Raf and ERK1/2 phosphorylation in cells that were pre-treated with PP2 prior to stimulation with FGF-2 or PMA. Western blotting of extracted proteins showed strong phosphorylation of Raf in FGF-2 treated samples compared with controls (Figure 5.11B). Conversely, Src kinase blockade abrogated Raf phosphorylation in both control and FGF-2 treated samples. However, Src inhibition had no effect on Raf activation in the presence of PMA, suggesting that PKC-mediated Raf activation is independent of Src kinase activity. These results indicate that Src kinase is important for activation of Raf-MAPK following FGF stimulated release of endothelial PlGF.

Src inhibition in the presence of FGF-2 suppressed ERK1/2 phosphorylation. This indicates that multiple signalling cascades, including those regulated by MAPK and PKC, work in parallel to facilitate FGF-2-mediated PlGF release from ECs.

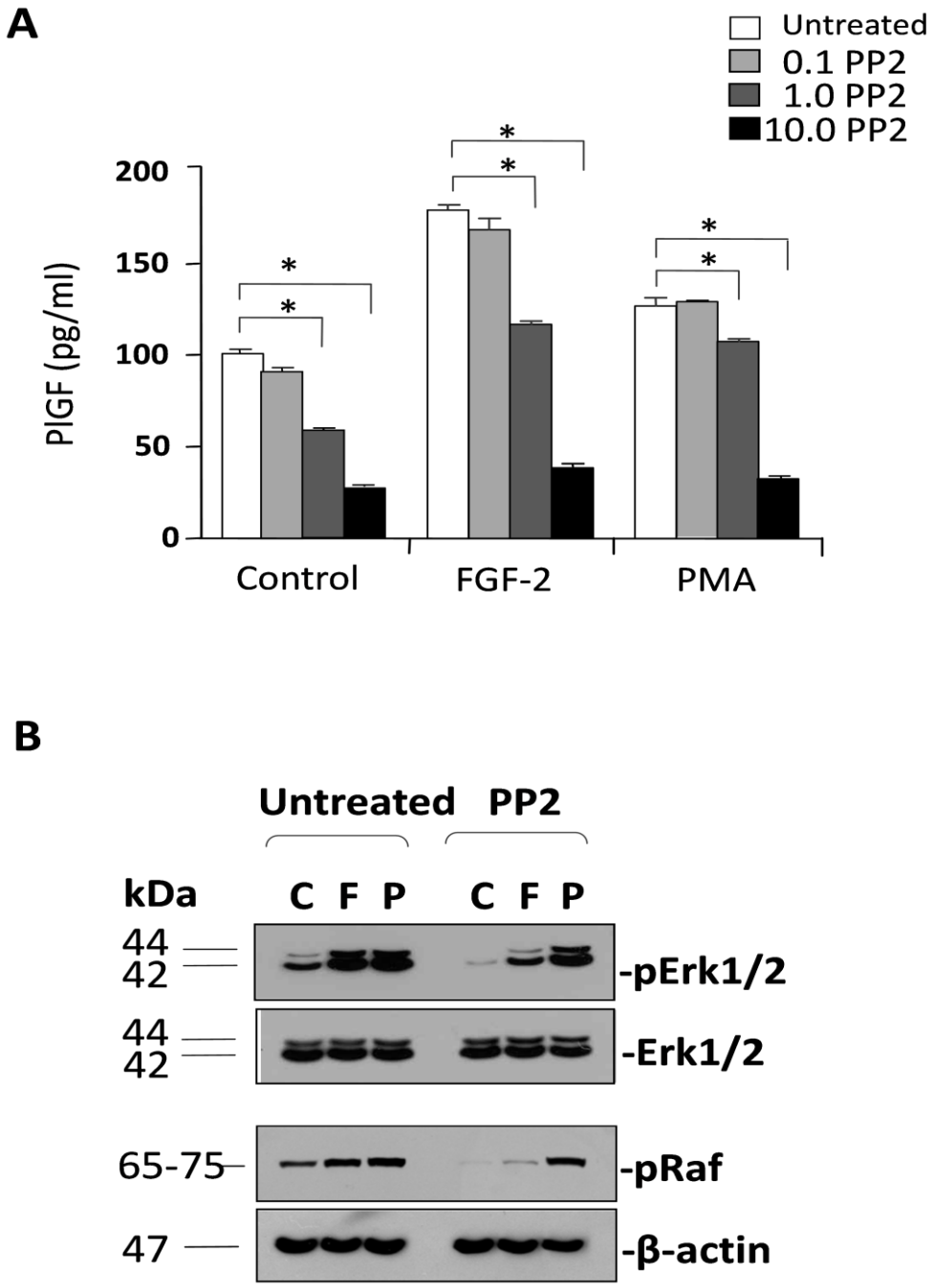


Figure 5.11 Src kinase involved in PlGF release from ECs. *(A) HMEC-1 were cultured in serum-reduced medium overnight and pre-incubated for 45 minutes with the pharmacological Src inhibitor PP2 (0.1 -10 µM), prior to the treatments with FGF-2 (10 ng/ml) or PMA (100 nM). Cell supernatants collected following 24 hour incubation and were analysed for PlGF by ELISA. Data are expressed as means ± SEM of measurements of 4 independent experiments performed in duplicates. (B) Representative Western blot showing the effect of Src kinase inhibitor, PP2 on ERK1/2 and Raf phosphorylation, following FGF-2 or PMA stimulation. HMEC-1 were pre-incubated with PP2 (10 µM) for 30 minutes followed by stimulation with FGF-2 or PMA for 15 minutes. 25µg of cell protein per treated sample was*

*run on a 10% SDS-PAGE. Activation of ERK1/2 was determined by Western-blot analysis using antibodies that recognized phosphorylated ERK1/2 (pERK or p42/44) or Raf (pRaf). The blots were re-probed with β-actin to demonstrate equal loading. C – Control, F – FGF-2 (10 ng/ml), P– PMA (100 nM). *P<0.05 compared to the control.*

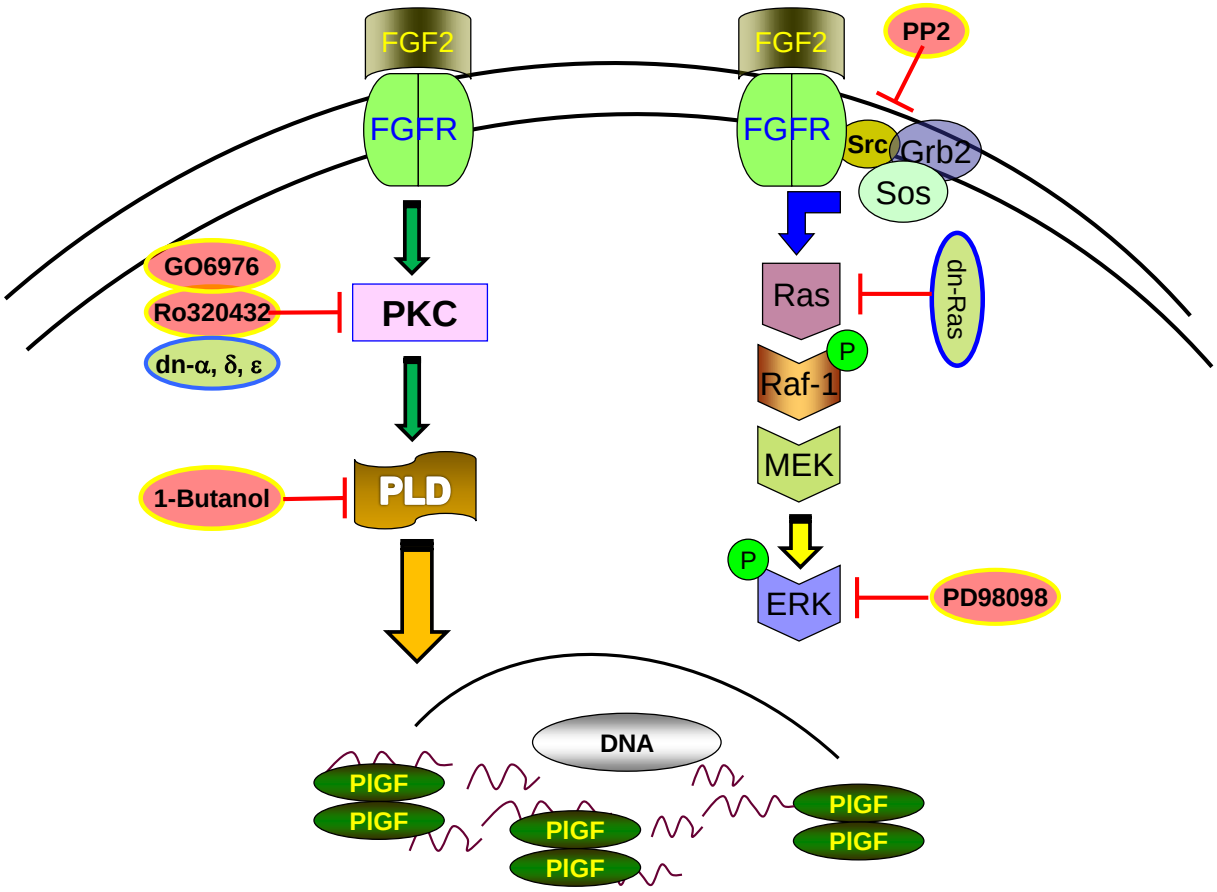


Figure 5.12 Pictorial representation of PlGF release involves Src kinase.

5.2.7 PKC and MAPK dependent pathways governing FGF-2 regulation

In order to confirm that PKC and MAPK regulate FGF-2-mediated PlGF release, we assessed whether PKC activation preceded MAPK phosphorylation. Raf and ERK1/2 activation were examined in the presence of diacylglycerol (DAG) kinase, which catalyses the phosphorylation of DAG. DAG is the physiological activator of PKC to phosphatidic acid (Houssa, de Widt et al. 1999) . Raf-1 activation was partially down-regulated under basal conditions, and in the presence of FGF-2 or PMA, suggesting possible cross-talk between the PKC and MAPK pathways (Figure 5.13). However, ERK1/2 phosphorylation was not affected by blocking DAG kinase, indicating PKC and MAPK are two independent pathways perhaps diverging from Raf-1 [Figure 5.7A]. To clarify the relationship between PKC and MAPK activation, we infected ECs with β-gal, PKCα^{DN}, Ras^{DN}, either individually or in combination, and treated the cells with FGF-2 or PMA. Cell lysates were subjected to Western blotting and probed for phosphorylated ERK1/2. PKCα ^{DN} alone did not affect ERK1/2 activation in the presence of FGF-2 (Figure 5.13). However, PMA induced ERK1/2 phosphorylation was blocked by 50%. PKCα^{DN}/Ras^{DN} completely inhibited ERK1/2. Conversely, PKCα ^{DN} enhanced basal activation of ERK1/2, suggesting under unstimulated conditions there might be negative regulation of ERK by PKCα.

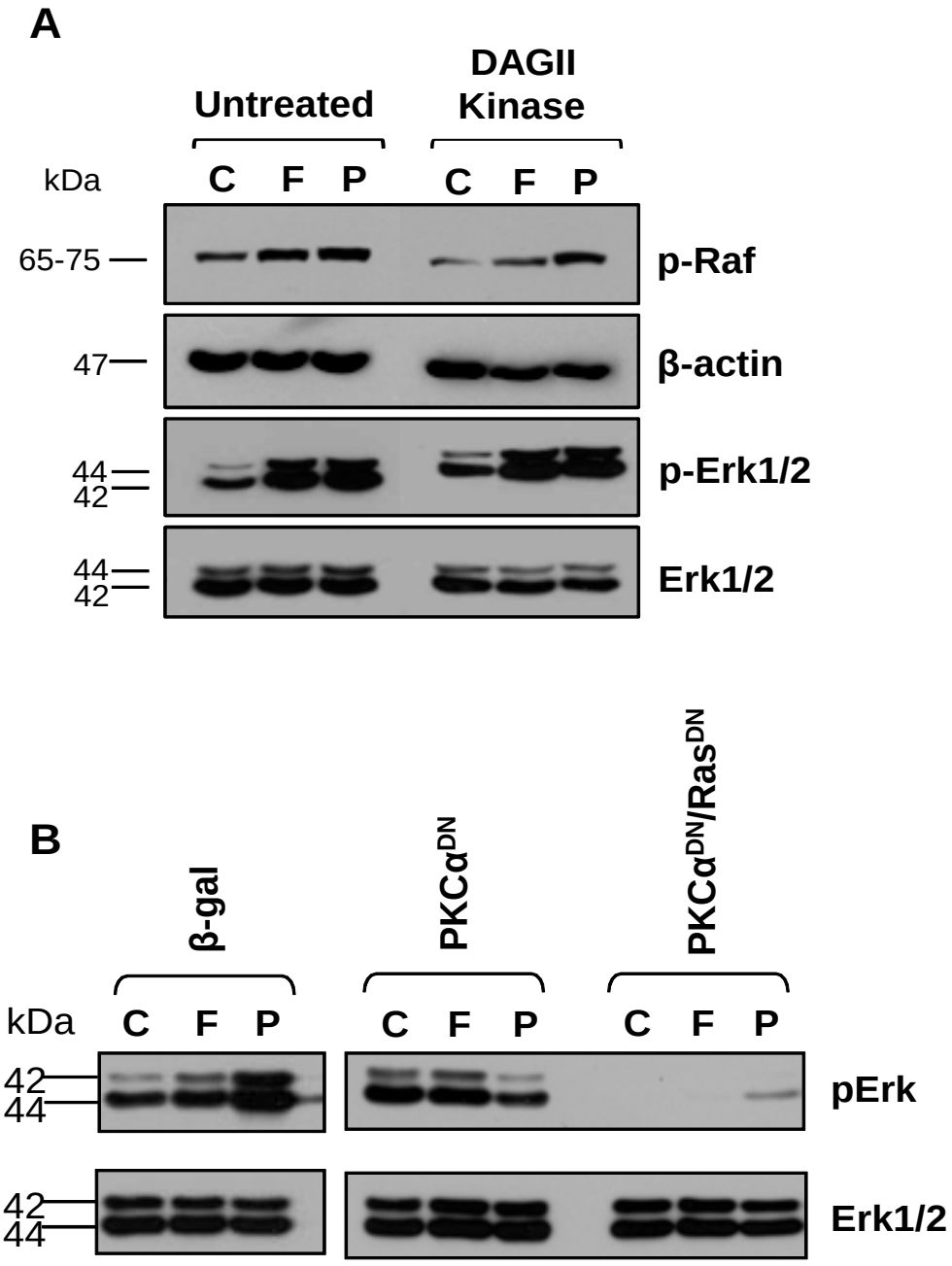


Figure 5.13 Representative Western blots showing the effect of DAG kinase inhibitor on ERK1/2 and Raf phosphorylation*, following FGF-2 or PMA stimulation. (A) HMEC-1 were pre-incubated with DAG kinase II (10 μM) for 30 minutes followed by stimulation with FGF-2 or PMA for 15 minutes. 25μg of cell protein per treated sample was separated by 10% SDS-PAGE. Activation of ERK1/2 was determined by Western-blot analysis using antibodies that recognized phosphorylated ERK1/2 (pERK or p42/44) or Raf (pRaf). The blots were re-probed with β-actin to demonstrate equal loading. C – Control, F – FGF-2 (10 ng/ml), P– PMA (100 nM). (B) HMEC-1 were infected with adenovirus expressing β-gal or PKCαDN or RasDN or PKCαDN/RasDN for 24 hours prior to stimulation with FGF-2 or PMA for 15*

minutes. 25µg of cell protein per treated sample was run on a 10% SDS-PAGE. Activation of ERK1/2 was determined by Western-blot analysis using antibodies that recognized phosphorylated ERK1/2 (pERK or p42/44) or total ERK1/2. The blots were re-probed with ERK1/2 to demonstrate equal loading. C – Control, F – FGF-2 (10 ng/ml), P– PMA (100 nM).

5.2.8 PlGF Release from ECs is regulated by PI3K

We determined whether PI3K contributed to FGF-2-induced PlGF release by modulating PI3K activity with the pharmacological inhibitor, LY294002 (Davies, Reddy et al. 2000). LY294002 (Figure 5.14A) markedly decreased PlGF levels in both the untreated and FGF-2 stimulated cells in a concentration-dependent manner. These data indicate that PI3K is required for both basal and FGF-2 mediated PlGF release. The effect of PI3K blockade on MAPK signalling was investigated in cells treated with LY294002 in the presence of FGF-2. Cell lysates were subjected to Western blot analysis and monitored for the phosphorylated forms of ERK1/2 and Raf-1 (Figure 5.14B). PI3K inhibition did not affect ERK1/2 activation [Figure 5.14B], indicating that PI3K and MAPK independently regulate FGF-2-mediated PlGF release from ECs (Ferby, Waga et al. 1996, Scheid and Duronio 1996). However, PI3K inhibition partially abrogated Raf-1 phosphorylation in the presence of FGF-2. This suggests that Raf-1 phosphorylation is regulated by PI3K in ECs

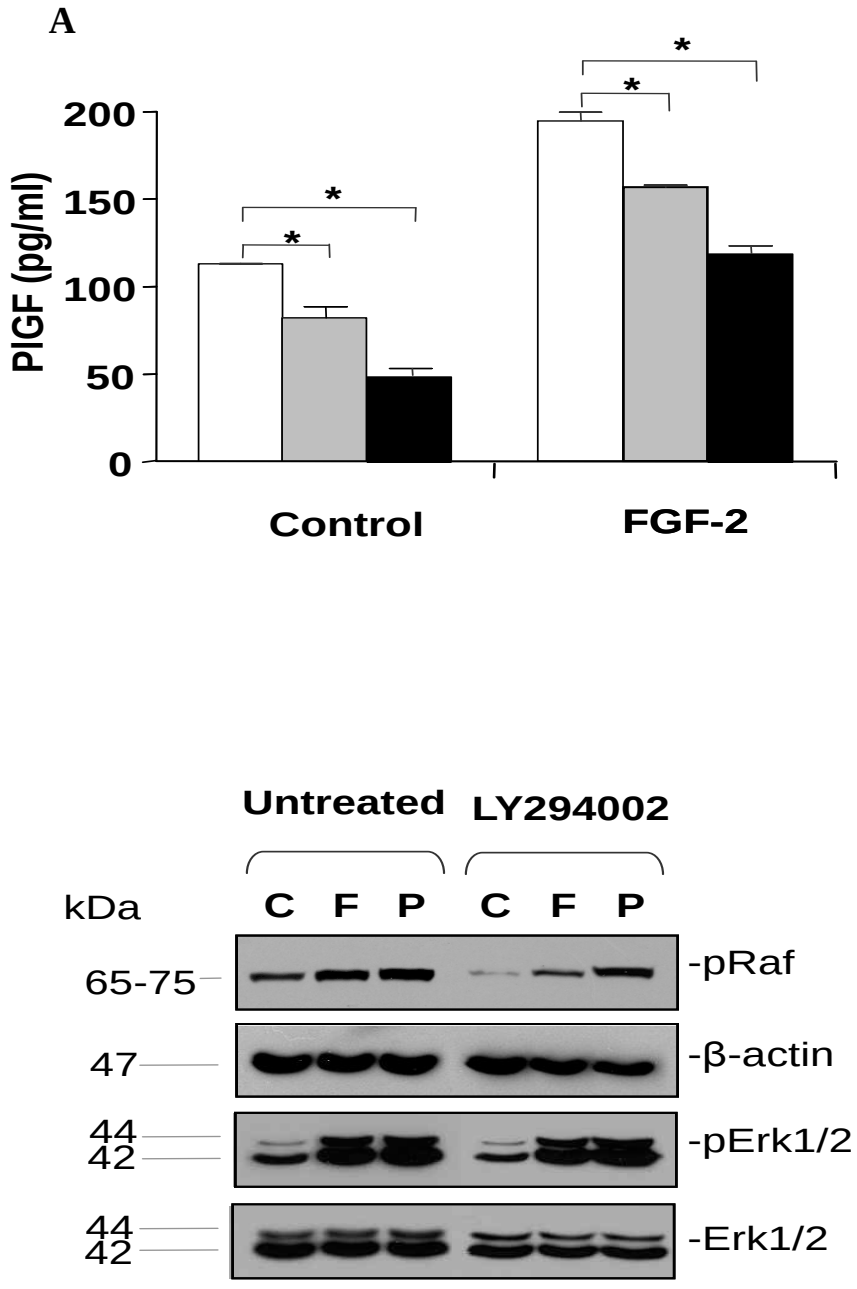


Figure 5.14 Ras and PI3 kinase activity are required for PlGF secretion from ECs. *HMEC-1 were cultured in serum-reduced medium overnight and pre-incubated for 45 minutes with pharmacological inhibitor of PI3 kinase LY294002 at indicated concentrations prior to the treatments with FGF-2 (10 ng/ml). Cell supernatants collected following 24 hour incubation and were analysed for PlGF by (A) ELISA. Data are expressed as means ± SEM of measurements of 4 independent experiments performed in duplicates. (B) A representative Western blot shows the effect of LY294002 on ERK1/2 and Raf phosphorylation following FGF-2 or PMA stimulation. HMEC-1 were pre-incubated with LY294002 (10 µM) for 30 minutes followed by stimulation with FGF-2 or PMA for 15 minutes. 25µg of cell protein per treated sample was run on a 10% SDS-PAGE. Activation of ERK1/2 was determined by*

*Western-blot analysis using antibodies that recognized phosphorylated ERK1/2 (pERK or p42/44) or Raf (pRaf). The same blots were re-probed with β-actin to demonstrate equal loading. C – Control, F – FGF-2 (10 ng/ml), P – PMA (100 nM). *P<0.05, compared to the control.*

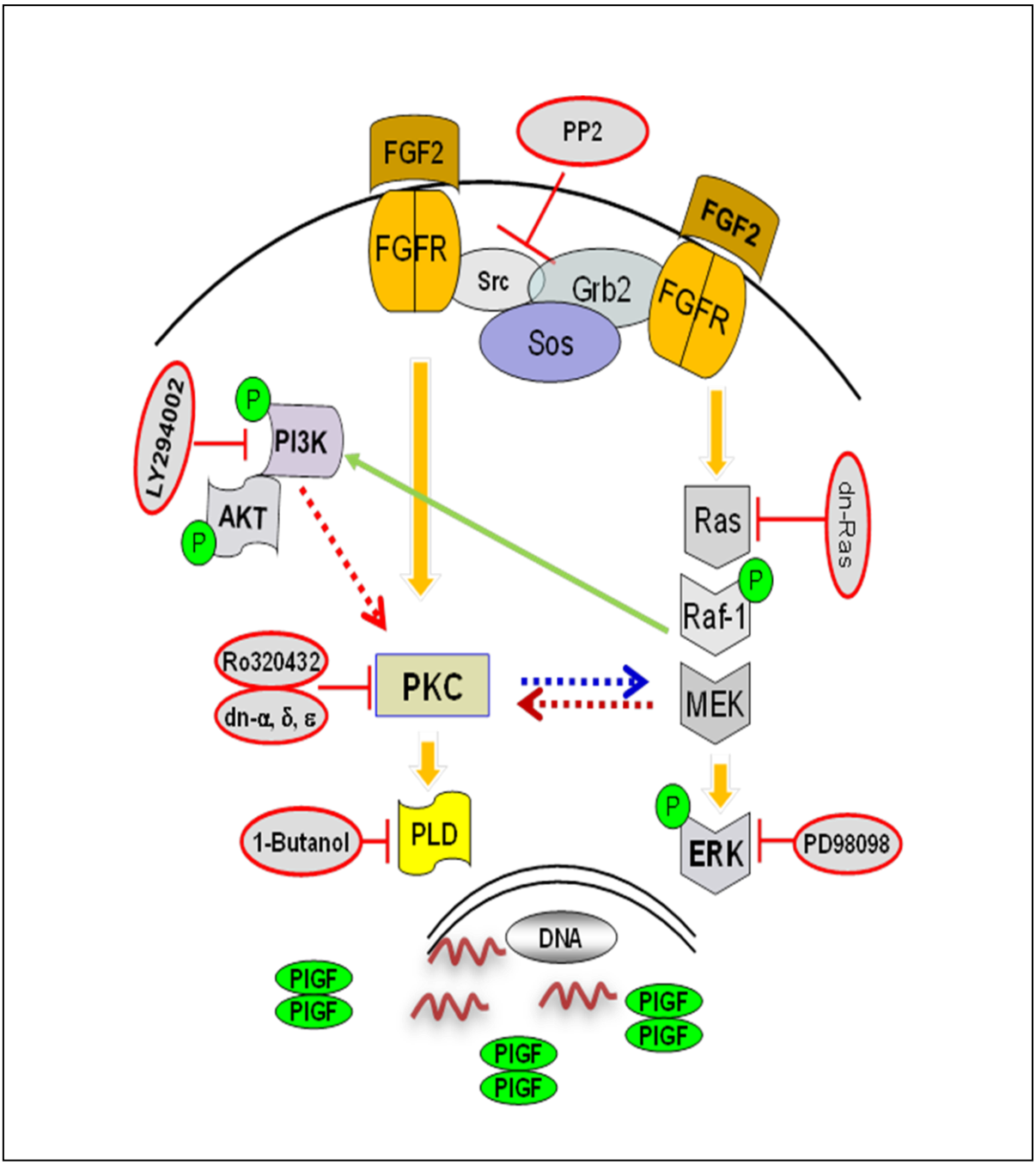


Figure 5.15 Multiple Signalling pathways FGF-2-mediated PlGF. *Diagrammatic representation of signaling pathways that may be involved in FGF-2-mediated PlGF release from ECs. As indicated by the diagram and as described by the data, PKC and MAPK pathways are central for the FGF-2-induced PlGF release. It is possible that the crosstalk between the PI3K and ERK pathways is mediated in part by PKC (Ahmed, Plevin et al. 1994). PKC signaling also is reported to occur directly with PI3K and upstream of Ras. Furthermore, direct interaction between MEK and PKC downstream of Ras is reported. Likewise, ERK2 (p42) is reported to signal to Raf-1 via a positive feedback mechanism (Cai, Smola et al. 1997).*

5.3 Discussion

Numerous lines of experimental evidence implicated that a number of cytokines and growth factors modulate angiogenesis *in vitro* and *in vivo* via an autocrine and/or paracrine mode of action. VEGF (Ferrara and Davis-Smyth 1997), FGF-2 (Basilico and Moscatelli 1992, Bikfalvi, Klein et al. 1997, Paleolog 2002) and more recently PlGF (Carmeliet, Moons et al. 2001) have been reported as potent angiogenesis inducers in physiological and/or pathological angiogenesis. Both VEGF and FGF-2 have a synergistic effect on angiogenesis (Asahara, Bauters et al. 1995, Kano, Morishita et al. 2005). Likewise, PlGF and VEGF have been demonstrated to play a synergistic role in pathological angiogenesis (Carmeliet, Moons et al. 2001). In the following chapters I have shown that FGF- 2-stimulated up-regulation of PlGF in ECs is, in part, responsible for its angiogenic activity.

FGF-2 induced a significant increase in PlGF mRNA levels compared to the control. PlGF protein levels, in response to FGF-2, followed a time and concentration-dependent mechanism. PlGF production in response to FGF-2 is via transcriptional activation of PlGF gene and *de novo* protein synthesis. In summary, up-regulation of PlGF protein was preceded by an increase of PlGF mRNA in a time-dependent manner in ECs when stimulated with FGF-2, unlike VEGF-induced PlGF release from ECs (Yao, Yang et al. 2005).

In this study, further evidence of the intracellular signalling pathways governing FGF-2-mediated PlGF release from ECs is detailed. PlGF release in response to FGF-2 occurs primarily via the activation of MAP kinase and PKC. A significant body of pre-clinical evidence and patient data demonstrated proof of the concept that both FGF-2 and PlGF have

valuable and the significant roles during tissue healing, in ischemic injury (Harada, Grossman et al. 1994, Horrigan, Malycky et al. 1999, Iwama, Uemura et al. 2006, Callaghan, Chang et al. 2008), for improving cardiac function, promoting survival post MI (Kardami, Detillieux et al. 2007, Liao, Porter et al. 2007, Roncal, Buysschaert et al. 2008) and in their efficacy to advance therapeutic angiogenesis (Harada, Grossman et al. 1994, Unger, Banai et al. 1994, Luttun, Tjwa et al. 2002, Pipp, Heil et al. 2003, Kolakowski, Berry et al. 2006).

FGF-2 strongly increased the phosphorylation of ERK1/2 and the MAPK inhibitor PD98059, which abrogated PlGF secretion induced by FGF-2 from ECs. In a similar manner, ERK1/2 inhibition significantly decreased PMA-induced PlGF levels as well. MEK1 and ERK1/2 were required for FGF-2-mediated protection of ECs from the intrinsic (stress) pathway of apoptosis (Alavi, Hood et al. 2003). MAPK activation by FGF-2 promoted cell proliferation and migration during cell development, and, like VEGF, inhibits endothelial cell apoptosis (Risau 1997)

p38 has been reported to negatively regulate the endothelial cell response in FGF-2-stimulated angiogenesis (Matsumoto, Turesson et al. 2002), accompanied by prolonged ERK1/2 MAPK activation and increased endothelial cell survival (North, Moenner et al. 2005). Moreover, it has been suggested that a direct interaction exists between phosphorylated p38 and ERK1/2, resulting in ERK1/2 deactivation (Zhang, Shi et al. 2001). Here we show, using the p38 inhibitor, SB203580, that p38 signalling is not involved in the release of PlGF protein from ECs. Activation of ERK1/2 and inhibition of p38 by FGF-2 is cardioprotective during ischemia-reperfusion injury in an isolated work-performing heart model of global low-flow ischemic mice (Dhalla 2010). Over expression of FGF-2 resulted in ERK1/2 activation, leading to protection from post-ischemic contractile dysfunction and MI (Dhalla 2010).

Additionally, FGF-2 also inhibits p38 activation during ischemia and reperfusion, leading to decreased cell death after ischemia-reperfusion injury (Dhalla 2010). These results indicate that activation of the MEK-ERK signalling pathway is necessary to mediate FGF-2-induced cardio-protection from both post-ischemic contractile dysfunction and myocardial infarction (Dhalla 2010). In addition to up-regulating VEGF expression, FGF-2 also amplified VEGFR-2, a selective receptor for ECs *in vivo* (de Vries, Escobedo et al. 1992), via a PKC and ERK1/2 MAPK-dependent mechanism in bovine retinal capillary ECs (BREC) (Hata, 1999 #613).

Pharmacological down-modulation of PKC activity dramatically inhibited the FGF2-induced increase in PlGF secretion to basal levels. Similarly, PMA (PKC agonist) showed a significant decrease in the levels of PlGF in the presence of PKC inhibitors. Selective down-regulation of PKC α, δ and ε, by using validated PKC dominant-negative (PKC-DN) constructs over-expressed in adenoviruses, established the roles of PKCα, δ and ε in FGF-2-induced PlGF release from ECs. á-APP modulator [á-APP], the specific activator, induced only marginal increase in PlGF when compared to the other PKC activators. In this respect, it must be observed that unlike FGF-2, VEGF-induced PlGF release from ECs was reported to be via a post-transcriptional mechanism involving the activation of PKCβ (Yao, Yang et al. 2005).

Pathophysiological studies in diabetic rats implicated PKCβ and PKCδ in hyperglycaemia-induced vascular dysfunction (Inoguchi, Battan et al. 1992) and PKCε in muscle insulin resistance (WHO 2013). PKCα and PKCβ have been implicated in cardiac hypertrophy (Wakasaki, Koya et al. 1997). In our study we found that PKCε isoform is involved in FGF-2-induced PlGF release. Thus a greater understanding of the functional diversity and

pathophysiological regulation of PKC isoforms mediating growth factor-induced regulation, especially pertaining to vascular complications, is likely to have important clinical and therapeutic benefits (Xu, He et al. 2005). This data so far gives supportive evidence that PKC might be involved in the increased production of PlGF in disorders pertaining to pathological angiogenesis.

An earlier study had reported that FGF-2-induced ERK1/2 phosphorylation is mediated by PKCε, which in turn enhanced anti-apoptotic proteins Bcl-X_L and XIAP, thereby promoting survival/chemo resistance of lung cancer cells (Pober and Sessa 2007). Mounting evidence in animal models corroborated the PKCε isoform as a central signalling hub, mediating early and late pre-conditioning responses and cardioprotection during reperfusion (Ping, Zhang et al. 1997, Qiu, Ping et al. 1998, Ping, Zhang et al. 1999). PKC ε was found to have a protective effect in myocardial preconditioning (Dorn, Souroujon et al. 1999). PKC's role in the activation of the MAP kinase cascade involves complex interaction mechanisms due to the specificities of the various PKC isoforms involved. However, constitutively active isoforms PKC α, δ and ε have been reported to activate MEK/MAPK via Raf (Cai, Smola et al. 1997, Schonwasser, Marais et al. 1998). The involvement of Ras (Leevers and Marshall 1992, Thomas, DeMarco et al. 1992, Mitra, Weber et al. 1993) and c-Raf (Schaap, van der Wal et al. 1993, Schaafhausen, Yang et al. 2013) in the PMA-induced activation of MAP kinases has been reported with rather paradoxical results. Using a series of PKC knock-out mutants and mutants with constitutive kinase activity, Ueda *et al* showed that PKC δ is actually involved in the signalling pathway from PMA to Raf-MEK-ERK activation that operates in a Ras-independent manner (Ueda, Hirai et al. 1996). FGF-2 administration prevents ischemia/reperfusion injury in animal models via activation of the protein kinase C (PKC) and

mitogen-activated protein kinase (MAPK) signalling cascades (Padua, Sethi et al. 1995, Padua, Merle et al. 1998, House, Melhorn et al. 2007).

PKC activation by FGF-2/FGFR via phosphatidylinositol-phospholipase C (PI-PLCγ) has been reported in Chinese hamster ovary cells (Lundin, Ronnstrand et al. 2003). FGF-2 stimulates PLD signalling via PKC activation in ECs, independent of PLCγ (Ahmed, Plevin et al. 1994). However, in our study, blockade of PI-PLCγ enzyme by U73122 had only a modest effect on PlGF release induced by FGF-2. In contrast, PlGF secretion induced by FGF-2 was completely suppressed by PLD inhibition. These data are in line with previous reports (Ahmed, Plevin et al. 1994). Ahmed et al demonstrated in HUVEC that activation of PLD is dependent on prior activation of PKC (Ahmed, Plevin et al. 1994) indicating PKC to be upstream of PLD (Ahmed, Plevin et al. 1994). PLCγ or PLD activation leads to the production of second messengers that stimulate PKC (Nishizuka 1995, Kook and Exton 2005).

Blockade of DAG, a secondary messenger and activator of PKC (Al-Lamki, Wang et al. 2005) using DAG Kinase inhibitor, did not lead to alterations in ERK1/2 activity. Yet inhibition of PKC eliminated FGF-2-induced ERK1/2 MAPK phosphorylation in BRECs (Forsten-Williams, Chua et al. 2005). Moreover, in the same study that the MEK inhibitor, PD98059, completely inhibited MAPK phosphorylation, FGF-2-induced VEGFR-2 expression was only reduced by 70%, suggesting that although the majority of FGF-2-induced VEGFR-2 expression is via MAPK, other signalling pathways are also involved (Forsten-Williams, Chua et al. 2005).

In this study, FGF-2-mediated PlGF release from ECs is via PKC and MAPK pathways independently. These pathways are indispensable for FGF-2-mediated PlGF release, most probably with Ras as the point of convergence for these two predominant pathways.

This study demonstrates that PlGF release under basal conditions and in response to FGF-2 from ECs involves multiple signalling cascades. PlGF release from ECs under basal conditions involved Ras/Raf/MEK, PKC and PI3K pathways, whereas FGF-2-mediated PlGF release was MEK and PKC specific. Statins reduced the expression of angiogenic growth factors FGF-2, HGF and TGF-β in mouse osteosarcoma by blocking Ras/MEK/ERK and Ras/PI3K/Akt pathways (Tsubaki, Yamazoe et al. 2011). Intracellular magnesium $[Mg^{+}]_i$ concentration was increased in response to FGF-2 via tyrosine kinase/PI3K/PLCγ signalling pathways in HUVEC (Hong, Park et al. 2009). Low magnesium promotes endothelial dysfunction as a common pathogenic event associated in cardiovascular diseases, including hypertension, atherosclerosis, inflammation and thrombosis (Cines, Pollak et al. 1998, Maier, Malpuech-Brugere et al. 2004). It would therefore be interesting to study FGF-2 and PlGF levels in pathologies with low Mg^{+} that lead to endothelial dysfunction.

Further work investigating the inter-dependency of the signalling pathways and the transcriptional factors regulating FGF-2-mediated PlGF release from ECs is warranted. There are moderately few of the anti-angiogenic agents for the treatment of not only CVD, but human cancers too, and many more of them are at various phases of clinical trials for therapeutic purposes. Resistance to apoptosis is a hallmark of carcinogenesis, and the initiation of apoptosis has been a vital focal point in the advancement of preventive drugs. Statins can induce apoptosis by regulating several signalling pathways, including the raf-MAPK1-ERK pathways (Carmeliet and Storkebaum 2002). The pleiotropic effects of statins

are related to their interactions with diverse signalling pathways and targets; for instance, pravastatin suppressed cell proliferation of HUVECs through inhibition of cell cycle (Asakage, Tsuno et al. 2004) and cerivastatin is indicated to have inhibited endothelial cell migration (Vincent, Chen et al. 2001). Further dissection of the signalling pathways that play a vital role in improved cardiac outcome, such as PKC and MAPK in ECs in response to various endogenous growth factors and cytokines triggered by injury/ischemia, could further our understanding of the beneficial effect of statins on atherosclerosis and on cancer prevention as shown by clinical studies (Ivan, Kondo et al. 2001). However, most of these anti/angiogenic agents target the VEGF signalling pathway (Folkman 2007). There is experimental confirmation which indicates that the tumour vasculature could acquire drug resistance towards VEGF therapy by shifting to other growth factor-induced angiogenic signals (Hicklin and Ellis 2005). For example, anti-VEGF agents may perhaps lead to drug resistance in the RIP-Tag spontaneous mouse pancreatic islet tumour model by switching to FGF-2 and other factors-induced angiogenesis (Gullestad and Aukrust 2005). Thus, it is important to consider a combination therapy by blocking common enzymes in multiple signalling pathways triggered by a range of angiogenic factors.

These results indicate the differential regulation of effector molecules in FGF-2-induced PlGF release from ECs in contrast to VEGF-stimulated PlGF (Yao, Yang et al. 2005). This would enable advancements in identifying novel therapeutic strategies to either promote revascularization of ischemic tissues, or to inhibit angiogenesis in cancer and inflammatory disorders.

Chapter 6 FGF-2-induced PlGF production in ECs: FGF-2-mediated angiogenesis is PlGF dependent

6.1 Introduction

Despite considerable progress in the management of vascular insufficient ischemic diseases, such as CVD, PVD, MI and heart failure, a significant proportion of patients have symptoms refractory to medical treatment. Advanced disease with/without the existence of various co-morbidities (Review) (Deveza, Choi et al. 2012) make these individuals unsuitable for conventional revascularisation techniques or surgical interventions, such as percutaneous coronary intervention (PCI), coronary artery bypass surgery (CABG) (Deveza, Choi et al. 2012) or percutaneous revascularisation (Di Stefano, Limbruno et al. 2004) to re-establish blood flow to the heart, brain or limbs (Review) (Deveza, Choi et al. 2012). Ischemic neovascularisation in the adult is a summation of three processes: vasculogenesis, angiogenesis and arteriogenesis (Lekas, Lekas et al. 2006, Silvestre, Mallat et al. 2008), with the aim of promoting tissue repair and remodelling of the blood vessels to address acute and chronic ischemic insults. Capillary network growth (angiogenesis) and/or collateral artery growth (arteriogenesis) form the key factors in the adaptive process of preserving tissue survival and organ function, saving limbs and lives following arterial occlusion in the vascular provinces of the body. These processes represent the final targets of therapeutic neovascularisation aimed at providing an alternative treatment strategy. Driven by clinical demands, concerted efforts are being applied to investigate two distinct categories of vessel growth to reduce ischemia; angiogenesis and arteriogenesis (Heil, Eitenmuller et al. 2006) have augmented in recent years opening new lease on life for patients with ischemic disease (Reviews) (Di Stefano, Limbruno et al. 2004, Deveza, Choi et al. 2012).

Treatment modalities aiming to restore the impaired blood supply can either be a macrovascular repair (arteriogenesis; in the case of critical arterial stenosis or occlusion) or microvascular repair via angiogenesis, such as the event of an ischemic attack of the tissue. Several growth factors and cytokines drive the underlying mechanisms of these processes (van Royen, Piek et al. 2001, Takeda, Uemura et al. 2009, Wu, Wu et al. 2010) (Figure 6.1).

PlGF (Carmeliet, Moons et al. 2001, Luttun, Tjwa et al. 2002, Iwama, Uemura et al. 2006, Kolakowski, Berry et al. 2006) and FGF-2 (Unger, Banai et al. 1994, Laham, Chronos et al. 2000, Lazarous, Unger et al. 2000, Coenegrachts, Maes et al. 2010, Wu, Wu et al. 2010, Schmidt, Kharabi Masouleh et al. 2011) are reported to be both pro-angiogenic and pro-arteriogenic without any deleterious side effects, such as hypotension, oedema, hemangiogenesis, proteinuria, impaired wound healing, haemorrhage and thrombosis or endocrine dysfunction; side effects which normally accompany VEGF therapy (Oura, Bertoncini et al. 2003, Takeda, Uemura et al. 2009) (Kamba and McDonald 2007). As a result, PlGF and FGF-2 have grabbed the attention of several research groups for their contribution towards the restoration of blood supply and cardiac function thereby enhancing survival rates.

During acute myocardial infarction (AMI), the endothelium was reported to be the major site for elevated PlGF expression, rapidly producing PlGF within the infarcted myocardium (Iwama, Uemura et al. 2006) and positively correlated with >60% improvement in left ventricular ejection factor (LVEF). With the above results as a background, Takeda *et al.* studied the therapeutic effects of PlGF in a mouse model of AMI (Takeda, Uemura et al. 2009). Three-day intraperitoneal administration of recombinant human PlGF via an osmotic

minipump enhanced both angiogenesis and arteriogenesis, resulting in the reduction of infarct size, improved cardiac function and increased survival rates post AMI (Takeda, Uemura et al. 2009). Co-administration of recombinant human (rh) sVEGFR-1 with rhPlGF lowered the survival rate of mice compared to rhPlGF treatment alone (Takeda, Uemura et al. 2009). Although the molecular mechanisms and factors that trigger and lead to angiogenesis and/or arteriogenesis are diverse, blood vessel growth is broadly accepted to be solely dependent on the function of ECs (Carmeliet 2000, M 2011).

Consolidated data from our observations and that of previous reports (Yonekura, Sakurai et al. 1999, Fischer, Jonckx et al. 2007, Dewerchin and Carmeliet 2012, Kim, Cho et al. 2012) demonstrate ECs to be likely as an important source of PlGF. PlGF and its receptor VEGFR-1 have gained increasing attention for 'their contribution to pathologies. Several lines of reports established the critical role of PlGF in tumour angiogenesis and ischemic tissues through VEGFR-1 signalling on ECs, as well as in inflammatory-mediated mechanisms or enhancing recruitment of the endothelial progenitor cell (EPC) (Carmeliet, Moons et al. 2001, Luttun, Tjwa et al. 2002, Odorisio, Schietroma et al. 2002, Autiero, Luttun et al. 2003, Autiero, Waltenberger et al. 2003, Pipp, Heil et al. 2003, Rakic, Lambert et al. 2003, Li, Sharpe et al. 2006). Therapies targeting revascularisation of ischemic tissues by enhancing capillary and collateral vessel formation in the heart and limb ischemic tissues with PlGF treatment (Luttun, Tjwa et al. 2002, Pipp, Heil et al. 2003, Kolakowski, Berry et al. 2006), and inhibition of tumour angiogenesis, arthritis and atherosclerosis using anti-VEGFR-1 (Carmeliet, Moons et al. 2001, Luttun, Tjwa et al. 2002) have been the focus for researchers throughout the past decade. PlGF, via VEGFR-1 alone (Terman, Khandke et al. 1994), promotes monocyte

migration (Clauss, Weich et al. 1996), the release of NO (Bussolati, Dunk et al. 2001), wound healing (Iwama, Uemura et al. 2006) and prolongs survival and stability of capillary networks (Cai, Ahmad et al. 2003).

Considerable interest has also arisen from numerous pre-clinical studies demonstrating the ability of FGF-2 to restore cardiac function post-MI (Kardami, Detillieux et al. 2007, Liao, Porter et al. 2007). Trans myocardial or extraluminal administration of FGF-2, in animal models, improved heart function, reduced infarct size and increased blood flow to areas of ischemia (Harada, Grossman et al. 1994, Unger, Banai et al. 1994). Additionally, FGF-2 increased angiogenesis in the myocardium, improved systolic function (Harada, Grossman et al. 1994, Horrigan, Malycky et al. 1999, Iwatate, Miura et al. 2001) and improved collateral development in microembolised animal models (Iwatate, Miura et al. 2001). Loss of FGF-2 in mice impaired the process of MI repair (Schultz, Witt et al. 1999), whereas overexpression of FGF-2 preserved cardiac function by enhancing cell proliferation, scar contraction and ventricular function (Virag, Rolle et al. 2007). Endogenous FGF-2 contributes to the pro-angiogenic effect of ACE inhibitors in cultured microvascular coronary ECs (Donnini, Solito et al. 2006) and elevated levels of FGF-2 accelerates wound healing (Callaghan, Chang et al. 2008) by potentiating the recruitment of leukocytes (Zittermann and Issekutz 2006). Kanda *et al* reported that FGF-2-mediated angiogenesis is dependent on the activity of VEGFR-1 (Kanda, Miyata et al. 2004), as incubation of HUVEC with anti-VEGFR-1 blocking antibody inhibited FGF-2-mediated tube formation (Kanda, Miyata et al. 2004).

Basing on the accumulated evidence from several sources that both FGF-2 and PlGF promote tissue healing and cardiac function following ischemic injury, post-MI, and as a therapeutic angiogenesis and/or arteriogenesis promoting agent, we hypothesized that FGF-2-induced angiogenesis is dependent upon the release of PlGF from ECs. Detillieux *et al* in his review suggested that the usefulness of FGF-2 in human therapy is dependent upon our ability to selectively augment its endothelial effects, and reduce smooth muscle effects (Pintucci, Steinberg et al. 1999, Detillieux, Sheikh et al. 2003), which can also be relevant to PlGF; PlGF has a direct impact on ECs, SMCs, macrophages and fibroblasts that express VEGFR-1 (Dewerchin and Carmeliet 2012).

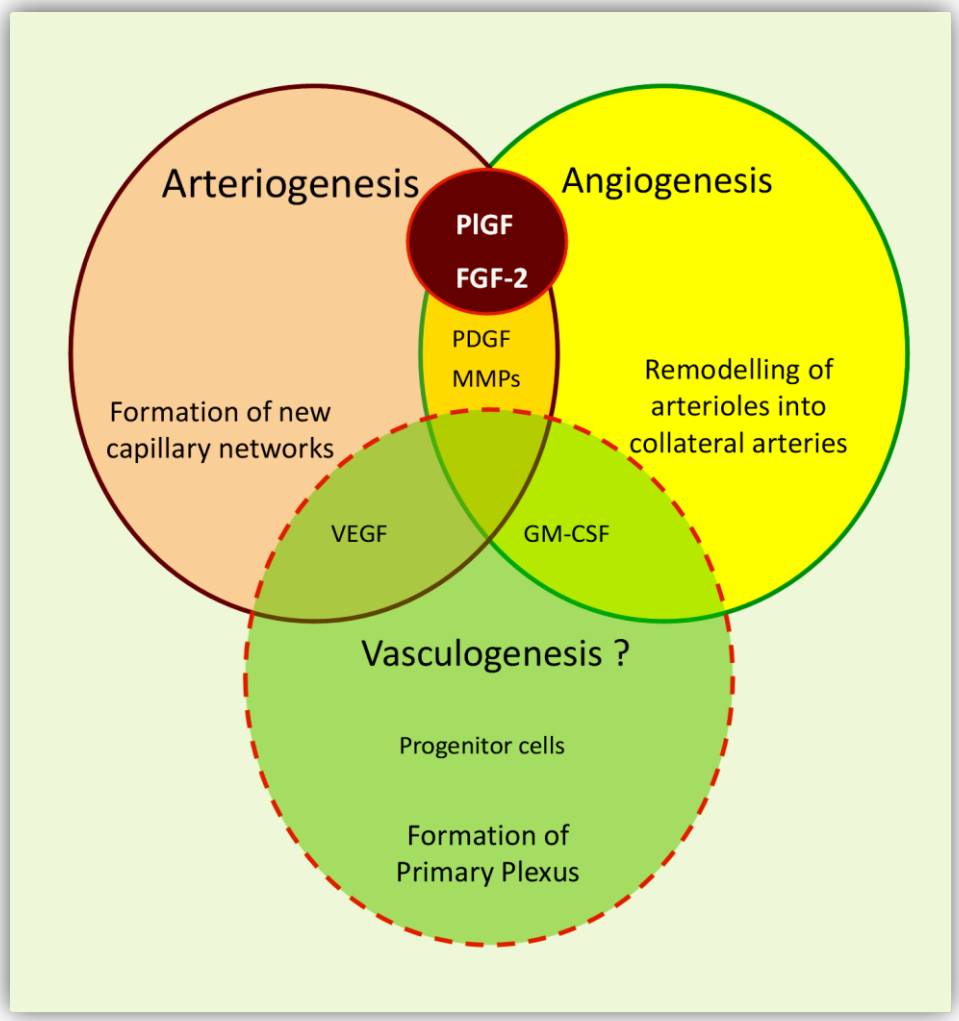


Figure 6.1 Factors involved in vasculogenesis, angiogenesis and arteriogenesis. *Three types of vessel growth, angiogenesis, arteriogenesis and vasculogenesis, are represented. PlGF and FGF-2 are both angiogenic, and arteriogenic and stimulate both processes. Furthermore, this image emphasises the overlap between angiogenesis, arteriogenesis and vasculogenesis. It is currently unknown whether vasculogenesis is operative in the process of vessel growth in response to obstructive arterial disease. PlGF involved in mobilisation of progenitors from the bone marrow is perhaps necessary for vasculogenesis. Factors that induce angiogenesis via the proliferation of ECs, whereas factors stimulating arteriogenesis additionally induce proliferation of smooth muscle cells as well. Adapted from Van Royen et al. Cardiovasc Res 2001;49:543-553 (van Royen, Piek et al. 2001).*

Although extensive work has been undertaken to examine the clinical significance of PlGF in animal models and human studies, via the loss of function and gain of function studies as a

disease-modifying candidate (Review) (Dewerchin and Carmeliet 2012), little is known about the factors that regulate endothelial PlGF. While we were investigating the factors that modulate PlGF expression in ECs, VEGF was reported to induce production of PlGF via a post-translational mechanism (Yao, Yang et al. 2005). The current chapter investgates whether the effects of FGF-2 on endothelial PlGF in an immortalised cell line (HMEC-1 Figure 5.1, 5.2)) are comparable to a primary one (HUVEC). More importantly, this chapter substantially explores if FGF-2-stimulated angiogenesis required PlGF expression.

6.2 Results

6.2.1 FGF-2 promotes PlGF production in HUVECs

To determine the response of endothelial PlGF in the presence of angiogenic growth factors, HUVEC were treated with VEGF, FGF-2 or PMA for 24 hours. Cell supernatants were collected and assayed for PlGF release by ELISA. VEGF induced PlGF levels in agreement with the findings of Yao and colleagues (Yao, Yang et al. 2005). A two-fold increase in the PlGF levels was observed in the presence of FGF-2 (Figure 6.2), which was comparable to the effect of VEGF. As VEGF is readily produced by ECs (Figure 6.2), PMA, a PKC activator (Furstenberger, Berry et al. 1981), was used as a positive control as per the requirement. PMA at an optimal concentration of 100nM (approximately 62 ng/ml) was used in this study (PMA concentration graph in Appendix II).

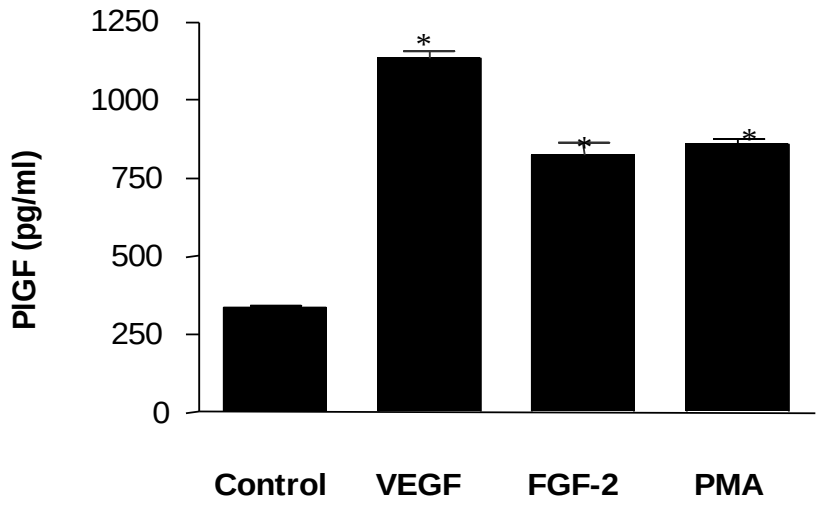


Figure 6.2 FGF-2-induced release of PlGF from ECs. *HUVEC were seeded at a density of 1.5 x 105 per well in 1% gelatin coated 24 well plate, and incubated overnight in RPMI containing 5% FCS. Cells were then stimulated with either VEGF [10 ng/ml], FGF-2 [10 ng/ml], or PMA [100 nM]. Following a 24-hour incubation supernatants were collected and PlGF measured by ELISA. Data expressed in pg/ml of PlGF and are mean [± SEM] of at least three independent experiments performed in triplicate. *P<0.05 vs. control.*

6.2.2 FGF-2-mediated PlGF release is concentration-dependent

To investigate whether FGF-2-induced PlGF release was concentration-dependent, HUVEC were treated with increasing concentrations of FGF-2 for 24 hours. A concentration-dependent rise in the levels of PlGF release in cell supernatants was observed in response to the increasing concentrations of FGF-2. PlGF secretion reached statistical significance after addition of 10, 50 or 100 ng/ml of FGF-2 compared to the untreated cells. The addition of 10 ng/ml FGF-2 caused a significant (two- to three-fold) increase in PlGF levels in both HUVEC (Figure 6.3) and was therefore chosen for subsequent experiments.

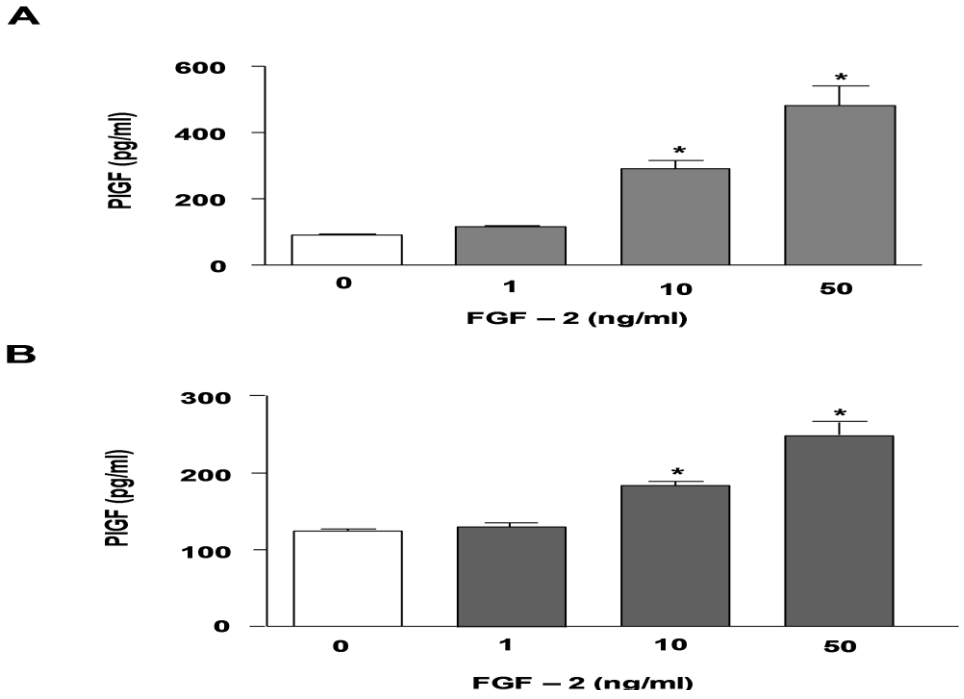


Figure 6.3 FGF-2 up-regulates PlGF release in a concentration-dependent manner. *HUVEC were cultured in serum-reduced medium and incubated overnight. ECs were stimulated with increasing concentrations (1, 10, 50 ng/ml) of FGF-2 for 24 hours. The conditioned media was collected and assayed for PlGF by ELISA. The data represent the mean (± SEM) of at least 4 independent experiments, performed in duplicate. * $p<0.05$ vs. Control.*

6.2.3 FGF-2 induces PlGF expression in ECs in a time-dependent manner

To investigate the kinetics of FGF-2-induced up-regulation of PlGF protein, HUVECs were treated with FGF-2. PlGF levels were measured at various time points, starting instantly, i.e. from 0.25 hours up to 24 hours to monitor for detailed post-treatment effects of FGF-2 on endothelial PlGF. Differences in the levels of PlGF release between FGF-2 treated and control cells became apparent after 8 hours. The maximum difference between FGF-2 treated and untreated cells was observed at 16 hours (Figure 6.4A). The optimal time of 24 hours was therefore selected for subsequent experiments unless specified. Real-time PCR analysis of RNA from HUVEC treated with FGF-2 for various time periods revealed that an increase in PlGF mRNA expression preceded the rise in PlGF protein secretion (Figure 6.4B). Moreover, a significant increase was observed in FGF-2-induced PlGF mRNA levels at 8, 16 and 24 hours compared to its control. Cells were incubated overnight in low serum medium, followed by fresh medium with or without FGF-2 before collecting RNA samples at different time points for real-time PCR. It is noteworthy to mention that PlGF protein stimulation by FGF-2 is significant at 16 hours. Therefore, it appeared that FGF-2 may regulate PlGF expression at a transcriptional level.

Experiments in this study concluded at 24 hours due to several reasons:

1. HUVEC are fragile and sensitive primary culture cells that require full medium with high serum concentrations. In this study, HUVEC is grown in medium supplemented with 5% serum. Adding extra serum or fresh medium could bring about radical changes in cell functions.
2. Opting to add a fresh medium for the experiments to run beyond 24 hours may contribute to artefacts in the results.

3. Most importantly, average doubling time for HUVEC and HMEC-1 is between 24-30 hours.

Bearing in mind these essential limitations regarding the cell types (HUVEC, HMEC-1) used extensively in this study, for subsequent experiments 24 hours' time point is chosen unless otherwise specified.

To further confirm the transcriptional regulatory effect of FGF-2 on PlGF levels, cells were treated with actinomycin D, a transcription inhibitor (Sobell 1985) (Figure 6.5A), or cycloheximide, an inhibitor of protein biosynthesis (Figure 6.5B), for three or six hours in the presence of FGF-2. As these treatments abrogated PlGF expression, it confirmed our assertion that the requirement of transcription for PlGF release in response to FGF-2 was critical. As expected, the basal levels and FGF-2-induced PlGF protein levels reduced by actinomycin D and cycloheximide; inhibitors of *de novo* RNA synthesis and *de novo* protein synthesis, respectively. This inhibition in PlGF suggests that PlGF production likely to be transcriptionally regulated.

Collectively, these results show that FGF-2-induced PlGF release is concentration-dependent through transcriptional activation of PlGF gene and *de novo* protein synthesis.

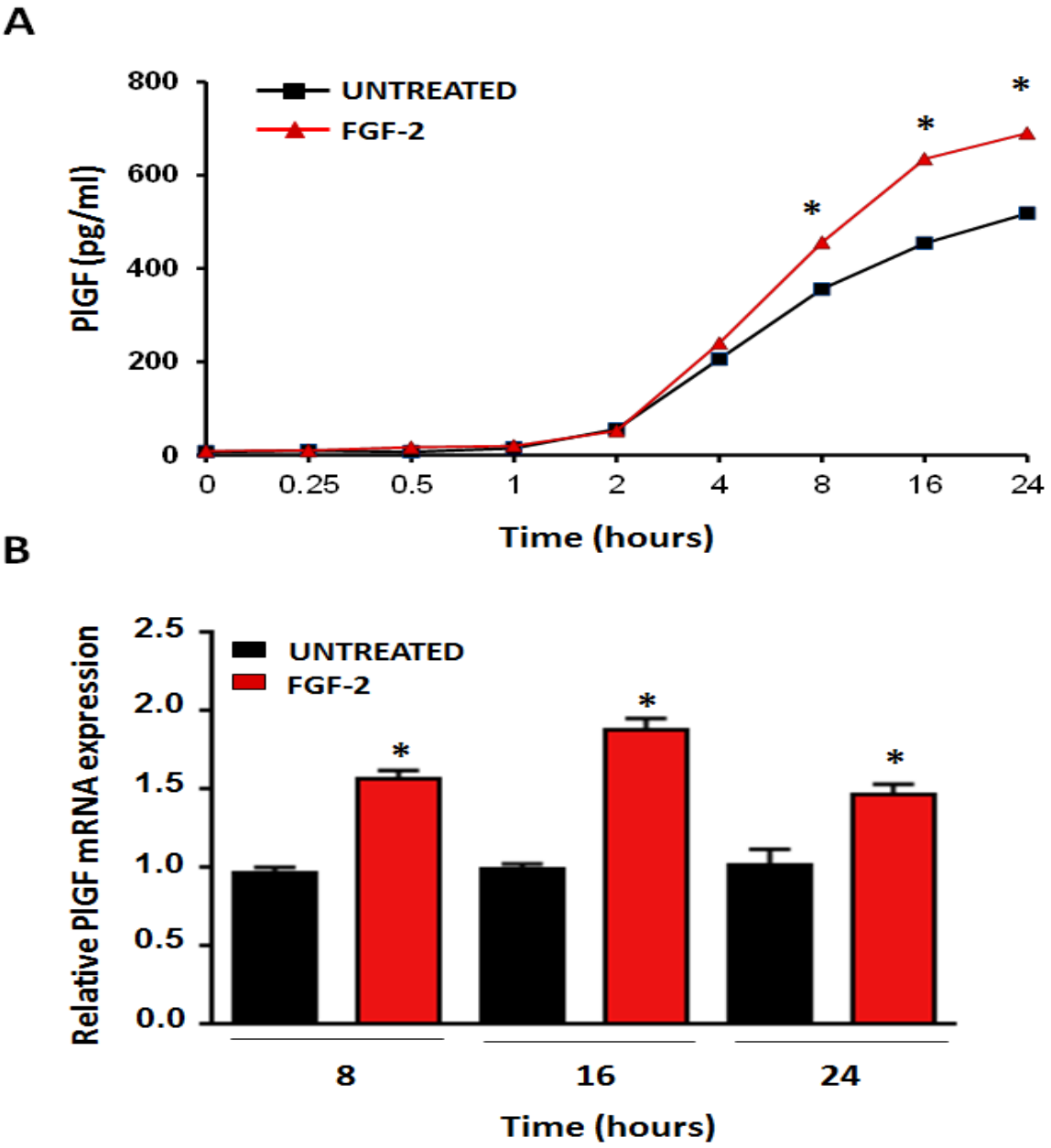


Figure 6.4 Kinetics of the FGF-2-induced release of PlGF in cultured ECs. *[A] Time-dependent PlGF protein release from HUVEC in response to FGF-2 [10 ng/ml] stimulation. Cell supernatants from conditioned medium were collected at indicated time points and PlGF levels measured by ELISA. [B] FGF-2-induced PlGF mRNA expression in a time-dependent manner. HUVEC were stimulated with FGF-2 (10 ng/ml) and mRNA subjected to quantitative real-time PCR for PlGF expression analysis. PlGF mRNA values are normalized to β-actin gene expression and presented as mean (± SEM) of three separate experiments performed in duplicate. * $p<0.05$ vs Control.*

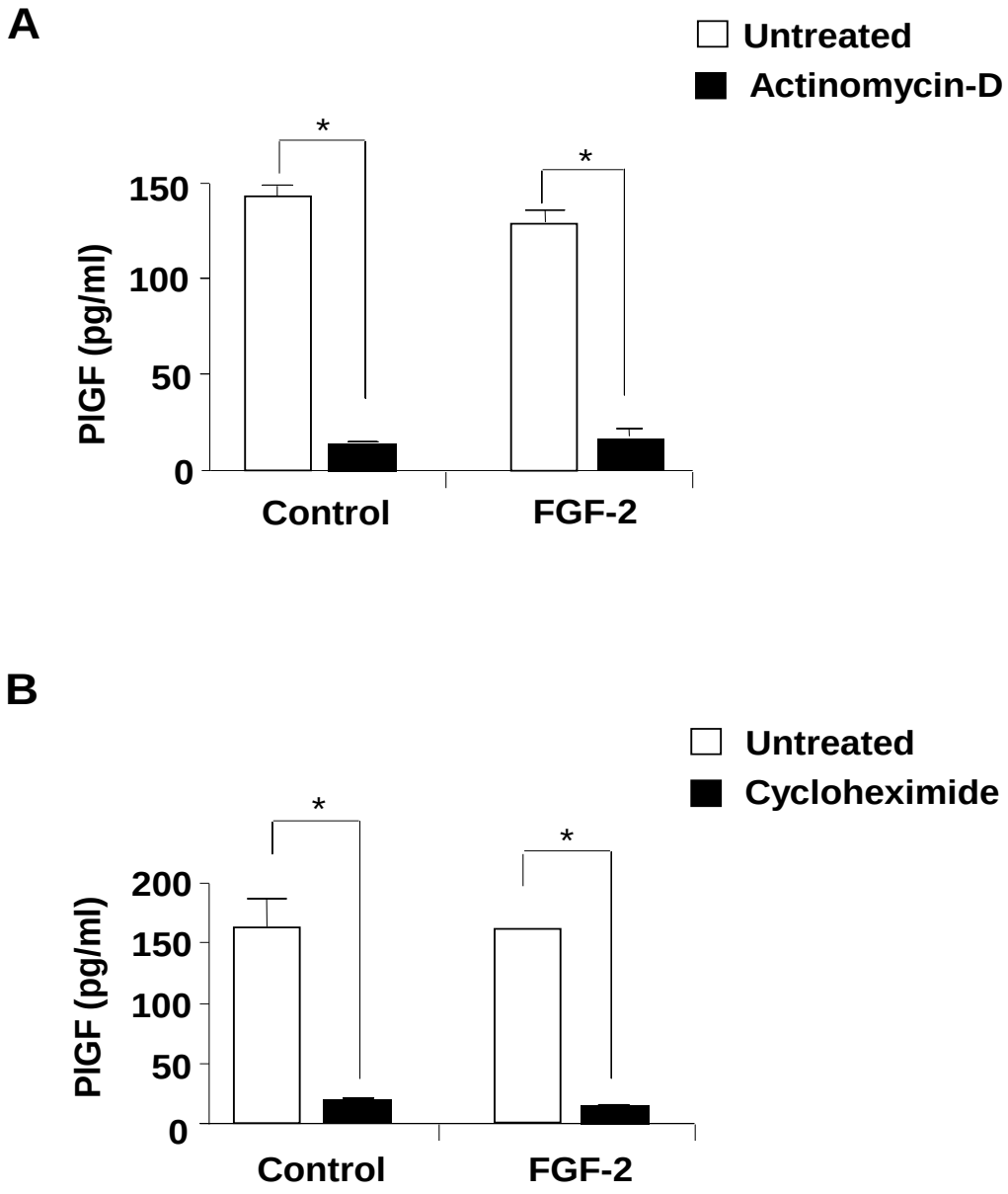


Figure 6.5 Kinetics of the FGF-2-induced release of PlGF in cultured ECs. *HUVEC were pre-incubated with (A) actinomycin-D [1 µg/ml] or (B) Cycloheximide [1 µg/ml] and then stimulated with FGF-2 [10 ng/ml] for 6 hours. Cell supernatants from conditioned medium collected at indicated time points and PlGF levels measured by ELISA. Data are mean [±SEM] of three separate experiments performed in duplicate. * p<0.05 vs Control.*

6.2.4 Functional Studies

6.2.4.1 FGF-2-induced in vitro tube formation is PlGF dependent

FGF-2-mediated *in vitro* capillary morphogenesis of HUVEC is reported to require VEGFR-1 (Kanda, Miyata et al. 2004). Previous work in our laboratory reported that PlGF enhances endothelial cell survival and sustained tube formation (Cai, Ahmad et al. 2003). To establish the requirement of PlGF for FGF-2-mediated endothelial tube formation, PlGF was knocked-down by PlGF siRNA in HUVEC and the cells were subjected to *in vitro* tube formation assay.

6.2.4.1.1 Validation of PlGF gene silencing in HUVEC

PlGF gene silencing by PlGF siRNA validation was achieved by measuring PlGF levels in PlGF gene silenced cells (PlGF siRNA) to control group (Control siRNA). HUVEC from both control group and PlGF siRNA samples were supplemented with 0.2 % or 20 % FBS for up to 24 hours, and cell supernatants from conditioned medium collected at 6, 12 and 24 hours were subjected to PlGF ELISA for PlGF protein levels (Figure 6.6). The PlGF siRNA completely attenuated PlGF expression (Figure 6.6) confirming the usage of PlGF gene silenced cells for loss of function.

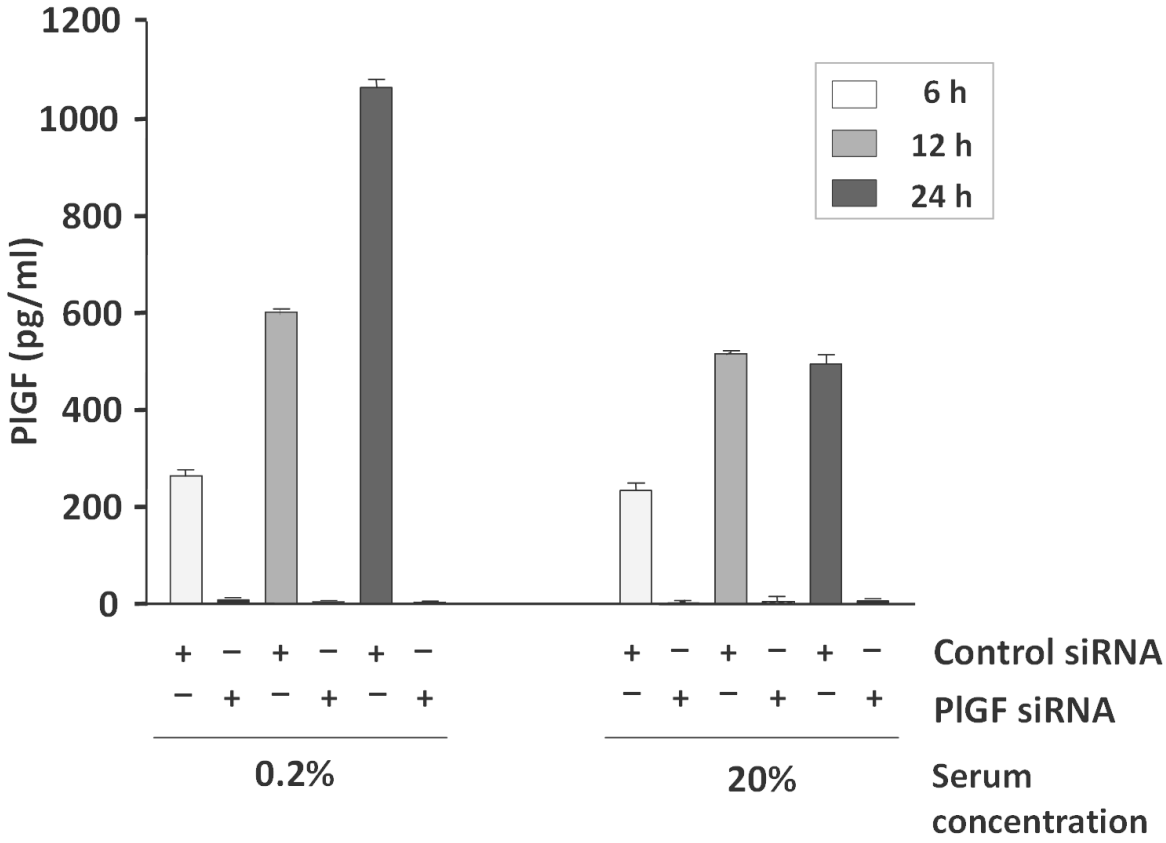


Figure 6.6 Validation of PlGF gene silencing in HUVEC. *PlGF expression attenuated in cell supernatants transfected with PlGF siRNA in HUVEC under 0.2 and 20 % serum conditions at various time points indicated. Cell supernatants assayed for PlGF by ELISA. Data are mean [±SEM] of three separate experiments performed in duplicate.*

6.2.4.2 In vitro tube formation assays in PlGF gene silenced endothelial cells

The siRNA-transfected cells were plated on growth factor-reduced Matrigel-coated wells, and FGF-2 was added to the appropriate wells. Endothelial tube-like structures were measured after 3 (Figure 6.7), 7 (Figure 6.8) and 24 hours (Figure 6.9) incubation.

HUVEC treated with FGF-2 triggered a two-fold increase in capillary-like structures, at 7 and 24 hours, compared to control wells. The ability of FGF-2 to form tubules was impaired in PlGF gene silenced cells (Figures 6.8, 6.9). Quantitative analysis showed an approximately two-fold decrease in total tube length in PlGF knock-down cells in the presence of FGF-2 at 7 and 24 hours compared to the control (Figures 6.8, 6.9). Graphical representation of the tube formation assay in total tube length (μm/field) measured in five random fields is displayed on the respective images at 3, 7 or 24 hours. These results clearly indicate the requirement of PlGF for FGF-2-mediated *in vitro* tube formation.

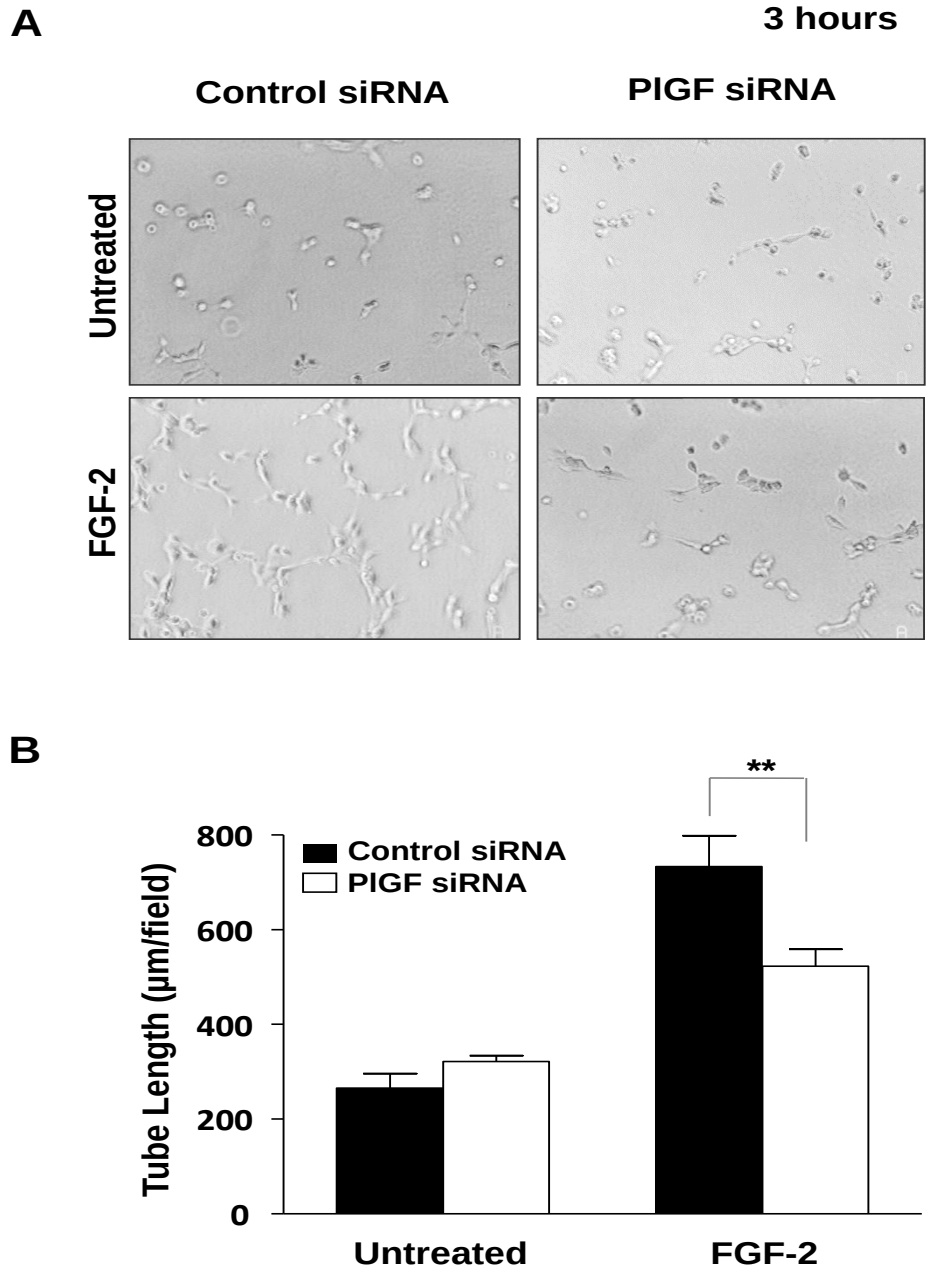


Figure 6.7 PlGF is required for FGF-2-induced in vitro tube formation in ECs. *HUVEC transfected with Control or PlGF siRNA and plated onto a six-well plate in MCDB 131 medium containing 5% FCS, and incubated overnight. The cells were trypsinised and 6x104 per well were seeded on growth factor reduced-matrigel in a 24 well plate. FGF-2 [10 ng/ml] was added to the appropriate wells. Mean total tube length calculated in five random fields per well.(A)Photomicrographs for 3 hours and (B) quantitative analysis demonstrating FGF-2- induced endothelial cell capillary network attenuation in PlGF-silenced cells. Data expressed as the mean ±SEM of at least three independent experiments in duplicates. **p<0.05vs Control.*

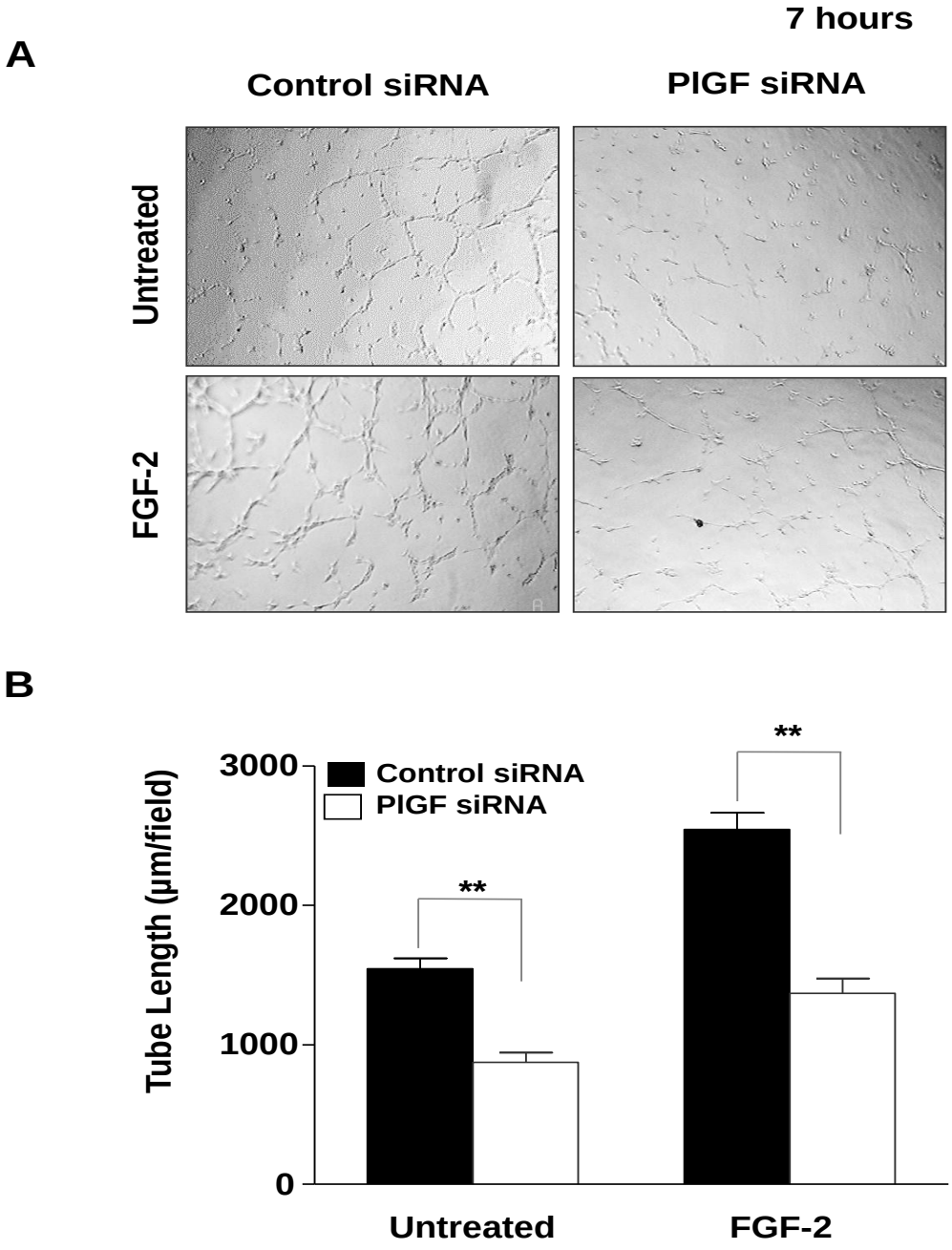


Figure 6.8 PlGF is required for FGF-2-induced in vitro tube formation in ECs. *HUVEC transfected with Control or PlGF siRNA and plated onto a six-well plate in MCDB 131 medium containing 5% FCS, and incubated overnight. The cells were trypsinised and 6x104 per well were seeded on growth factor reduced-matrigel in a 24 well plate. FGF-2 [10 ng/ml] was added to the appropriate wells. Mean total tube length calculated in five random fields per well.(A)Photomicrographs for 7 hours and (B) quantitative analysis demonstrating FGF-2- induced endothelial cell capillary network attenuation in PlGF-silenced cells. Data are expressed as the mean ±SEM of at least three independent experiments in duplicates. **p<0.05vs Control.*

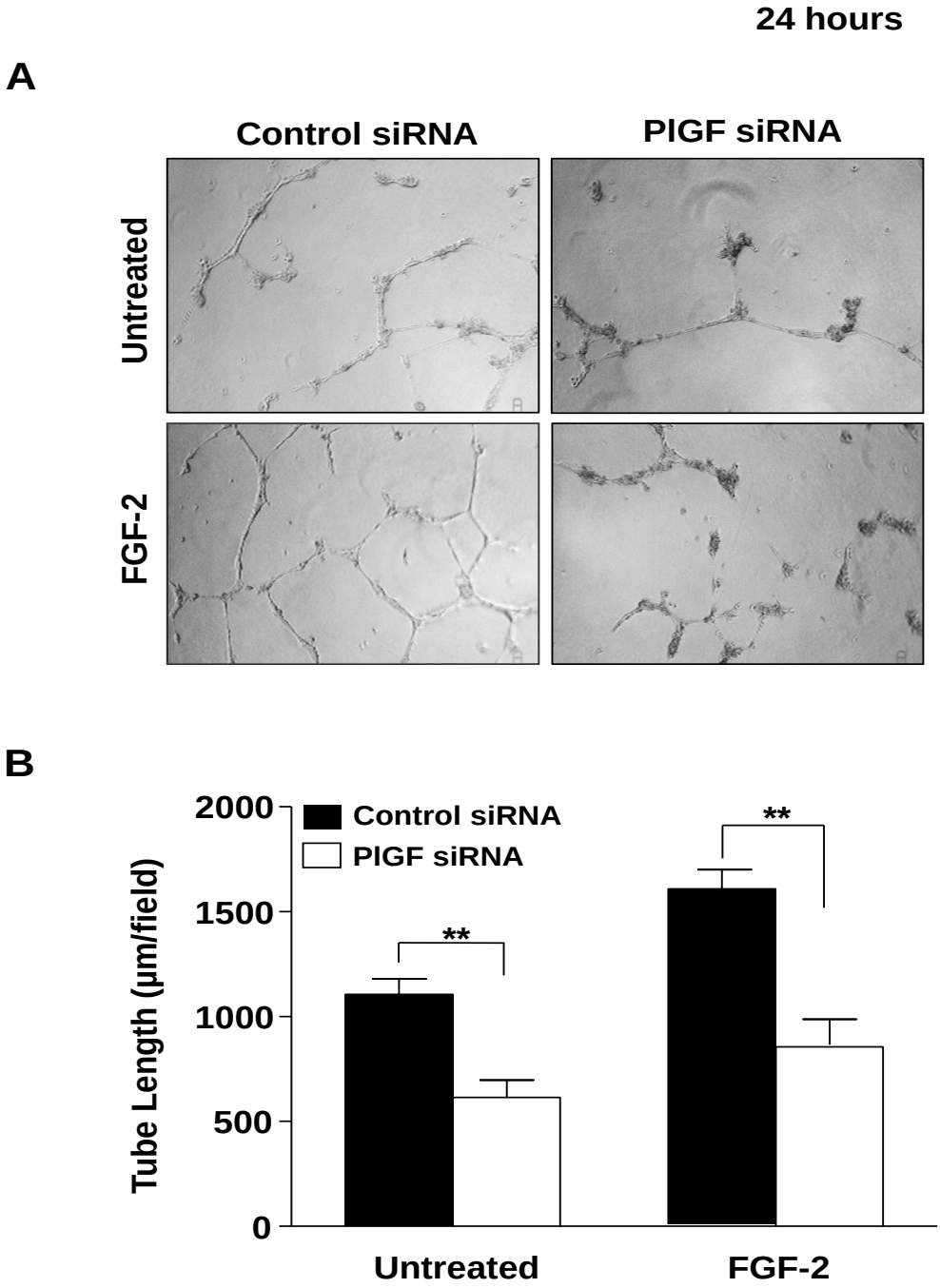


Figure 6.9 PlGF is required for FGF-2-induced in vitro tube formation in ECs. *HUVEC transfected with Control or PlGF siRNA and plated onto a six-well plate in MCDB 131 medium containing 5% FCS, and incubated overnight. The cells were trypsinised and 6x104 per well were seeded on growth factor reduced-matrigel in a 24 well plate. FGF-2 [10 ng/ml] was added to the appropriate wells. Mean total tube length calculated in five random fields per well.(A)Photomicrographs for 24 hours and (B) quantitative analysis demonstrating FGF-2- induced endothelial cell capillary network attenuation in PlGF-silenced cells. Data are expressed as the mean ±SEM of at least three independent experiments in duplicates. **p<0.05vs Control.*

6.2.5 FGF-2-induced angiogenesis is PlGF-dependent: Mouse explant Aortic Ring Assay

To further assess whether FGF-2-mediated angiogenesis is dependent on PlGF expression, the mouse aortic ring assay in $plgf^{-/-}$ mice was performed. Aortic ring assay is a valuable model that bridges the gap and combines the advantages of *in vivo* and *in-vitro* models, giving further insight into endothelial migration and angiogenic sprouting (Nicosia 2009). Aortic rings were prepared as previously described (Lewis E.C 2009, Nicosia 2009) from the resected dorsal aortas from PlGF-deficient ($plgf^{-/-}$), or from the age-matched corresponding wild-type (WT) mice, and embedded in collagen gels in the presence of FGF-2 (2 and 10 ng/ml). After seven days, vascular sprouting around the aortic explants were examined using an inverted phase-contrast microscope [Figure 6.10A]. Endothelial outgrowth and tube formation were well established after seven days in FGF-2 treated WT, and this observation was taken as an indication to end the experiment. In contrast to FGF-2-induced microvessels in the WT aortic rings, $plgf^{-/-}$ aortic explants had no significant angiogenic response following FGF-2 stimulation [Figure 6.10A]. Quantification of the total sprout lengths in each of the experimental conditions showed FGF-2 at both 2 and 10 ng/ml concentrations induced a three- and two-fold increase respectively in the sprout length in WT aortic explants, compared to its control. Notably, 2 ng/ml of FGF-2 induced 55% more of vessel sprouts compared to 10 ng/ml of FGF-2 in WT aortic explants. This variation, when compared to *in vitro* tube formation (Figures 6.8, 6.9), could be explained due to the presence of the mixed population of native cells, i.e. SMCs and pericytes that may well interact through paracrine mechanisms (Lewis E.C 2009, Nicosia 2009) under the chemically defined culture conditions designed in this experimental settings. Also, with regards to the endothelial sprouting, the maximum migration, i.e. the furthest away distance migrated from each aortic ring, was also reduced in

plgf $^{-/-}$ cultures. Collectively, these data propose PlGF necessary for FGF-2-mediated angiogenesis.

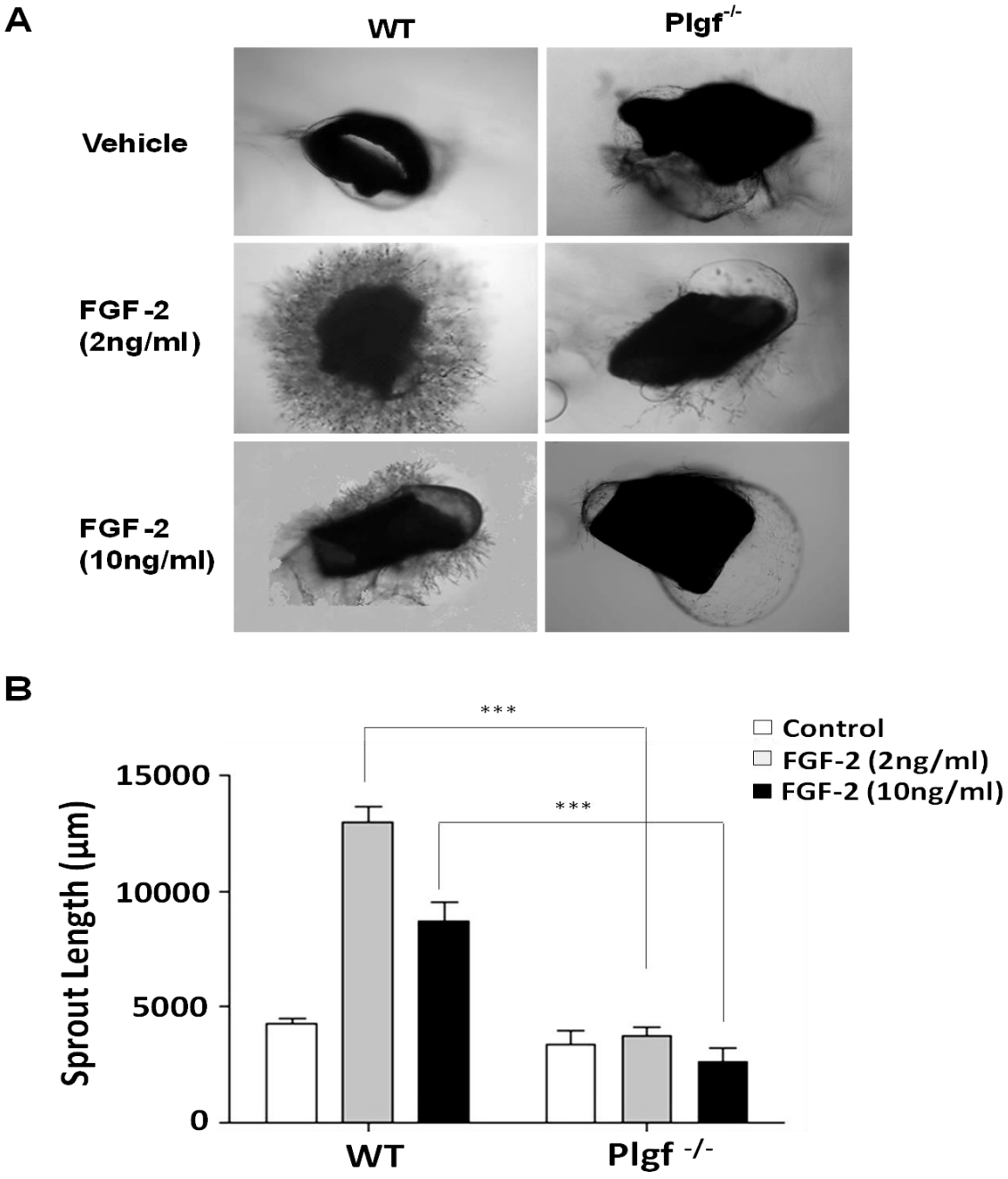


Figure 6.10 FGF-2-mediated angiogenesis is dependent on PlGF expression *[A] Representative photomicrographs of seven-day-old collagen gel cultures of mouse aorta. Mouse aortic explants extracted from wild-type (WT) and PlGF-knockout (Plgf-/-) mice were washed in PBS, sectioned into ~1 mm rings, and embedded in collagen in tissue culture wells. The tissues were coated with collagen with minimum air bubbles. After collagen solidification, the aortic ring cultures were maintained in the absence or presence of exogenous FGF-2. [B] Quantification of the total sprout length is expressed in µm. Data expressed as the mean ±SEM of four independent experiments repeating each condition in duplicate ***p<0.0001 vs control.*

6.3 Discussion

This is the first study to establish a direct link between the angiogenic activities exerted by FGF-2 and PlGF in ECs. The important contribution of the study is to demonstrate substantial evidence suggesting that FGF-2-induced angiogenesis requires PlGF. We believe that this evidence would add to the working concept of PlGF and FGF-2 contributed neovascularisation processes, and help to promote to improve therapies further.

6.3.1 FGF-2 induces PlGF expression in ECs

FGF-2-induced a significant increase in the secretion of PlGF protein levels in a concentration- and time- dependent manner, in macro- as well as micro- vascular ECs. These results are not consistent with findings of Yao and colleagues, which concluded that FGF-2 did not modulate the level of PlGF release from HUVEC significantly (Yao, Yang et al. 2005). In this study (Figure 2, page 1229, (Yao, Yang et al. 2005)), it was evident that FGF-2 increased PlGF levels by approximately three-fold when compared to the control. Furthermore, differences in cell culture method and conditions perhaps contributed to this discrepancy (Appendix IV). Yao et al allowed HUVEC grown to confluence in EGM-2MV medium (with no documentation of cell numbers) in 12-well plates, and then switched to DMEM containing 10% calf serum (Yao, Yang et al. 2005). Moreover, only a fixed concentration of FGF-2 (25 ng/ml) was used (Yao, Yang et al. 2005). On the other hand, in the current study a fixed number (1.5×10^5) of ECs per well in a 24-well plate were incubated for 24 hours in 5% FBS-containing RPMI and analysed for PlGF levels. These culture conditions were maintained throughout the study unless mentioned otherwise. There is a possibility that the change of growth medium and high serum concentrations used in Yao study, known to induce PlGF levels (Chapter 7, Figure 7.6), may not have allowed FGF-2 to

elicit any further additional effect on PlGF levels in their study (Yao, Yang et al. 2005). Besides, we have examined in more detail the effect of FGF-2 on PlGF in ECs; we show a gradual increase in PlGF levels in response to increasing concentrations of FGF-2 in both HUVEC and HMEC-1. VEGF upregulation was reported to occur via a post-transcriptional mechanism (Yao, Yang et al. 2005). We observed FGF-2 induce a significant increase in PlGF mRNA levels compared to the control. Unlike VEGF-induced PlGF release from ECs (Yao, Yang et al. 2005), FGF-2-induced PlGF secretion was dependent on PlGF gene transcription. PlGF production in response to FGF-2 is via transcriptional activation of PlGF gene and *de novo* protein synthesis, i.e., up-regulation of PlGF protein was preceded by an increase of PlGF mRNA, in a time-dependent manner in ECs when stimulated with FGF-2. Intramyocardial delivery of FGF-2 in rat models reduced infarct size and improved cardiac function, when assessed at 4-24 hours post-MI (Jiang, Padua et al. 2002), which are within the time periods of FGF-2-induced PlGF levels in the current study. Although we demonstrate that the FGF-2 induced PlGF increase occurs via a transcriptional regulation mechanism in ECs, these results could be consolidated by identifying transcription factors that mediate the effects of FGF-2 on PlGF gene promoter.

FGF-2 induces VEGF expression in vascular ECs through autocrine and paracrine mechanisms (Seghezzi, Patel et al. 1998). Though FGF-2 manifests variety of effects on EC function and gene expression, the findings in this study raises the possibility that both FGF-2 and PlGF may regulate neovascularization in an autocrine and paracrine manner by modulating expressions of other potent angiogenic factors, such as VEGF.

In the present study we demonstrate FGF-2 induced an increase in PlGF levels by up to two- to four-fold (200 - 400 pg/ml) when compared to the control (100 pg/ml). this was

demonstrated in macro- and micro- vascular ECs that may be sufficient to exert a biological effect. In AMI patients' plasma, PlGF levels ranged between 30 to 120 pg/ml, and higher levels of PlGF positively correlated with subsequent improvement in LVEF in the chronic phase; six months after the onset of AMI when compared to patients without improvement (Iwama, Uemura et al. 2006). These findings raise the possibility to speculate that FGF-2-induced PlGF concentrations may enhance VEGF-driven angiogenesis through the unique cross-talk between VEGFR-1 and VEGFR-2. Moreover, PlGF concentration at 100 pg/ml stimulated DNA synthesis in VEGFR-1 expressing ECs (Landgren, Schiller et al. 1998). In agreement with a previous report (Yao, Yang et al. 2005), VEGF and the PKC activator PMA both up-regulated PlGF protein levels secreted from the ECs. Unlike FGF-2, VEGF up-regulation of PlGF occurs via a post-transcriptional mechanism (Yao, Yang et al. 2005). Up-regulation of PlGF mRNA and protein was observed during angiogenesis in a number of metabolic, inflammatory disorders and tumours, including MI (Iwama, Uemura et al. 2006), diabetic retinopathy (Khaliq, Foreman et al. 1998), brain tumours (Nomura, Yamagishi et al. 1998) and ischaemic retinal disorders (Yamashita, Eguchi et al. 1999). Based on these reports and our findings in this chapter, it is tempting to speculate that the increase in PlGF protein and mRNA levels noted in these pathologies is perhaps up regulated by FGF-2, as FGF-2 expression has been reported in such disorders.(Riley, Savage et al. 1993, Fannon, Forsten-Williams et al. 2003, Presta, Dell'Era et al. 2005).

6.3.2 FGF-2-induced angiogenesis requires PlGF expression

Pre-clinical studies and patient data provide proof of the beneficial effects of PlGF or FGF-2, on post AMI pathology in the infarcted myocardium. The proposed effect works by enhancing tissue healing following ischemic injury, improving cardiac function and subsequent increase

in the survival rates (Laham, Sellke et al. 1999, Jiang, Srisakuldee et al. 2004, Iwama, Uemura et al. 2006, Kardami, Detillieux et al. 2007, Takeda, Uemura et al. 2009) (Tables 1.3, 1.4). Mice over-expressing PlGF in epidermal keratinocytes (under the keratin 14 promoter) exhibited a robust increase in dermal blood vessels, vascular permeability, in addition to increased inflammatory response (Odorisio, Schietroma et al. 2002, Oura, Bertoncini et al. 2003). Conversely, a lack of PlGF resulted in an inhibited and abbreviated inflammatory response, in concert with reduced inflammatory angiogenesis and oedema formation (Carmeliet, Moons et al. 2001, Luttun, Brusselmans et al. 2002). PlGF contributes to regulating the angiogenic switch, and its significant role in promoting aberrant angiogenesis in a variety of pathologies has gained increasing attention in the past few years. Unlike VEGF, FGF-2 is a pro-angiogenic growth factor that is well-tolerated and free of significant adverse effects on the restoration of cardiac function (Lazarous, Unger et al. 2000, Kardami, Detillieux et al. 2007, Liao, Porter et al. 2007). A series of animal studies and observations in patients with AMI reported that the up-regulation of PlGF is essential to facilitate restored cardiac function, and attenuate the adverse effects seen in the post-ischaemic heart (Table 1.3) (Iwama, Uemura et al. 2006, Kolakowski, Berry et al. 2006, Roncal, Buysschaert et al. 2008, Takeda, Uemura et al. 2009). We demonstrated that FGF-2-mediated *in vitro* tube formation was dependent on PlGF, by knocking down PlGF gene utilising the gene silencing approach. Complete inactivation of PlGF gene for up to 24 hours was achieved in cells incubated with different serum concentrations. A two-fold increase in PlGF levels under low serum conditions was observed when compared to high serum concentrations. These results indicate that PlGF is secreted by ECs in adverse or stressful conditions, probably to restore cell survival potential. This data is in line with previous studies from our laboratory, reporting

recombinant PlGF promoting long-term survival of ECs via PI3-kinase pathway (Cai, Ahmad et al. 2003).

A significant decrease (about 40-50 percent) in FGF-2 stimulated tube formation was observed in the absence of PlGF demonstrating the requirement for PlGF in this process. Kanda and colleagues showed that an anti-VEGF-A antibody reduced FGF-2-induced capillary morphogenesis (Kanda, Miyata et al. 2004). The addition of PlGF-1 restored anti-VEGF-A-mediated inhibition of capillary morphogenesis, proposing that VEGFR-1 signalling by endogenous VEGF-A may be required for FGF-2-mediated changes of EC shape (Kanda, Miyata et al. 2004). Although the Kanda *et al.* study compliments our results, there are few limitations in their study. We were unable to detect VEGF protein as well as mRNA in a range of ECs, including HUVEC, that were screened by ELISA and real-time PCR (Figure 4.2). Experiments to determine the optimal concentrations of VEGF or FGF-2 to elicit cellular responses were not documented in Kanda *et al* study. Moreover, Kanda *et al* used a higher concentration of VEGF (20 ng/ml) or FGF-2 (20 ng/ml) (Kanda, Miyata et al. 2004), in comparison to this study which elicited cellular responses in ECs, including the significant increase in PlGF levels at a lower concentration of FGF-2 (10 ng/ml). Fujii and colleagues demonstrated, in severe hind limb mouse ischemic model, that VEGF up regulation of endogenous PlGF expression required in FGF-2-mediated therapeutic angiogenesis (Fujii, Yonemitsu et al. 2008). Both FGF-2 and PlGF work in close association with each other during FGF-2-mediated therapeutic angiogenesis (Fujii, Yonemitsu et al. 2008). However, this study demonstrates no effect of FGF-2 on PlGF expression *in vitro* (Fujii, Yonemitsu et al. 2008). Although *in vivo* data of Fujii and colleagues support our findings, we believe that the differences *in vitro* findings might have been due to variations in cell culture methods, such as source of cells, isolation procedures, and initial cell culture conditions that are known

to influence the response to pro-angiogenic factors (e.g. VEGF, FGF-2) (Suparna Sanyal 2009). Some data, such as the growth medium and the passage number of the cells used were not documented (Fujii, Yonemitsu et al. 2008). In 2011, our group demonstrated a key finding that VEGF stimulates sVEGFR-1 expression from ECs *in vitro* and *in vivo* (Ahmad, Hewett et al. 2011), which raised questions about the findings of Kanda (Kanda, Miyata et al. 2004) or Fujii (Fujii, Yonemitsu et al. 2008) studies.

The advancement of the current study demonstrating FGF-2-mediated *in vitro* tube formation assay requires PlGF was further consolidated by *ex vivo* aortic ring assay findings performed on WT and *Plgf*$^{-/-}$ in the presence of FGF-2. Pericytes and vascular SMCs residing at the interface between the endothelium and the surrounding tissue serve to be the potential pro- or anti-angiogenic target complementary to the endothelium (Nicosia 2009).

This model provided invaluable information on the role of PlGF in FGF-2-mediated angiogenesis, as vessels that grow out from aortic ring assays recruit SMCs and pericytes are known to be the nearest possible comparison of neovessel formation *in vitro* settings to that of *in vivo.* Perhaps due to these causes FGF-2 at lower concentrations (2 ng/ml) induced almost double the number of sprouts in WT aortic rings when compared to 10 ng/ml treated WT aortic rings. Additionally, this response might have been due to a concerted effort of FGF-2 induced VEGF expression (Seghezzi, Patel et al. 1998) which is supported by the recent findings from our laboratory of the concentration dependent up regulation of sVEGFR-1 by VEGF (Ahmad, Hewett et al. 2011). More so, reduced sprouting was observed in *Plgf*$^{-/-}$ aortic rings in the presence of FGF-2, matching the findings of *in vitro* tube formation assay. Pericytes being the primary source of VEGF is stimulated by endothelial-derived NO (Reynolds, Grazul-Bilska et al. 2000). We therefore anticipated that a paracrine loop between

ECs producing NO and the pericytes which produce VEGF, in response to this NO may consequently coordinate and reinforce initial angiogenic activation. However, this was not the case; probably due to endothelial sVEGFR-1 expression being regulated by VEGF (Ahmad, Hewett et al. 2011).

Although we have demonstrated our findings with substantial evidence, the limitations, such as inconsistency in the handling of the aortic rings and the amount of surrounding tissue, can influence the vessel outgrowth. Furthermore, a number of variables, including the type of angiogenic stimulation, age, gender and the genetic background of the animals used, may influence the result as well (Zhu, Iurlaro et al. 2003). To overcome these setbacks, increasing the number of replicates of the current aortic ring assay with minimising the above variables would be the step to move forward.

Both endogenous and exogenous FGF2 induces VEGF expression in ECs via autocrine mechanism (Seghezzi, Patel et al. 1998). VEGF (Yao, Yang et al. 2005) and FGF-2 (Current study), enhance the expression of PlGF. Based on the above , we anticipated that FGF-2 mediated angiogenesis *in vitro* as well as *ex vivo* in the absence of PlGF should sustain EC functions via FGF-2-induced VEGF (Seghezzi, Patel et al. 1998). However, the major factor perturbing this concept is the recent contribution from our laboratory that VEGF up regulates sVEGFR-1 *in vitro* as well as *in vivo* (Ahmad, Hewett et al. 2011). Collectively, these findings suggest that FGF-2 directly induces PlGF and indirectly, via FGF-2, induced up regulation of VEGF. VEGF, in turn, may modulate PlGF levels via transcription (Current study) and post-transcriptional mechanism (Yao, Yang et al. 2005) respectively, and therefore demonstrating that PlGF is required for FGF-2 mediated angiogenesis. Future studies to

analyse PlGF, FGF-2 and VEGF compromise in endothelial function due to sVEGFR-1 secreted by VEGF in PlGF inactivation settings, is warranted.

In animal models, FGF-2 administered post ischemia/reperfusion injury mediated acute protection against cardiac dysfunction and tissue damage involved activation of PKC and MAPK signalling pathways (Padua, Sethi et al. 1995, Padua, Merle et al. 1998, House, Melhorn et al. 2007). FGF-2 and VEGF use distinct intracellular pathways to activate angiogenesis (Hood, Frausto et al. 2003). VEGF and αvβ5 or FGF-2 and αvβ3 cooperate to promote differential regulation and activation of the Ras-ERK pathway (Hood, Frausto et al. 2003). These distinct signalling molecules activated may play a role in the divergent vascular and survival responses elicited by FGF-2 and VEGF in inducing PlGF expression and its manifestation on cellular responses. Schematic illustration of PlGF, FGF-2 and VEGF interaction with the receptors activated to induce EC responses is represented in Figure 6.11. Elucidation of the expression of PlGF and its regulation in ECs may provide insights into the molecular mechanisms and the role of PlGF in aberrant angiogenesis, and its ability to reinstate normal vasculature in pathologies requiring neovascularisation, including cardiac restoration.

Although PlGF exerts cellular responses via VEGFR-1 (Yla-Herttuala and Alitalo 2003), it was also reported that angiogenic response to PlGF is indirectly mediated by VEGFR-2 (Roy, Bhardwaj et al. 2005). It might be that the loss of PlGF may have reduced the availability of VEGF to promote VEGFR-2 driven angiogenesis. The findings are presented here to unveil a part of the complexity of FGF-2-mediated angiogenesis requiring PlGF, leading to the vital question of VEGF contribution in this process.

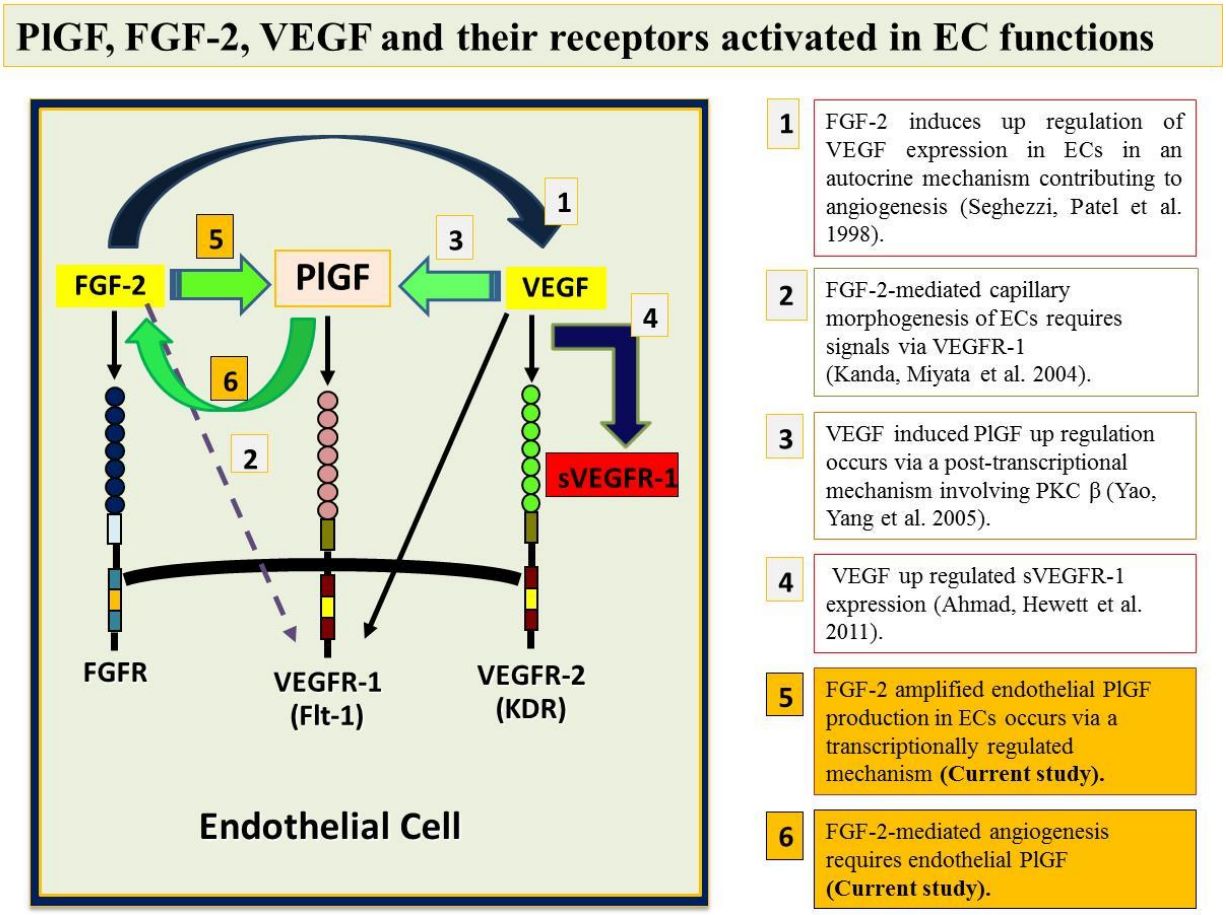


Figure 6.11 Schematic illustration of PlGF, FGF-2, VEGF and their receptors activated in EC functions. *1. FGF-2 induces up regulation of VEGF expression in ECs in an autocrine mechanism contributing to angiogenesis (Seghezzi, Patel, et al. 1998). 2. FGF-2-mediated capillary morphogenesis of ECs requires signals via VEGFR-1 possibly via Akt (Kanda, Miyata, et al. 2004). 3. VEGF-induced PlGF up regulation occurs via a post-transcriptional mechanism involving PKC β (Yao, Yang, et al. 2005). 4. VEGF up regulated sVEGFR-1 expression (Ahmad, Hewett, et al. 2011). 5. FGF-2-induced PlGF production in ECs occurs via transcriptional regulatory mechanism (Current study). 6. FGF-2-mediated angiogenesis is PlGF dependent (Current study).*

Additionally, as both VEGF and FGF-2 differentially regulate alpha integrin-mediated Ras-ERK signalling to elucidate angiogenesis (Hood, Frausto et al. 2003), it is advantageous to delineate the signalling mechanism governing FGF-2 induced PlGF release in ECs. The distinct signalling pathways involved in the induction of PlGF by ECs via VEGF (Yao, Yang et al. 2005) or FGF-2 (Chapter 5) could promote a better understanding of the interactions

or/and interdependencies that exist between these potent growth factors, to modulate the EC responses to either facilitate or inhibit angiogenesis.

Chapter 7- TNF-α inhibited PlGF release and its effect on endothelial cell functions

7.1 Introduction

Chronic inflammation is a hallmark of several inflammatory disorders and plays a key role in the pathogenesis of endothelial dysfunction (Murdaca, Colombo et al. 2012). Angiogenesis in the pathology of chronic inflammation is a highly coordinated process, involving a complex network of chemical signals and cell interactions. A wide variety of cells involved in the inflammatory processes release numerous factors that act directly or indirectly on vascular ECs, resulting in "switching on" or "switching off" the angiogenic process (Folkman 1995, Carmeliet and Jain 2000).

PlGF is an angiogenic stimulator and a strong pro-inflammatory mediator, which modulates the inflammatory process in a number of ways (Clauss, Weich et al. 1996, Bottomley, Webb et al. 2000, Selvaraj, Giri et al. 2003), for instance by the recruitment of inflammatory cells, pericytes and SMCs (Adini, Kornaga et al. 2002, Fischer, Jonckx et al. 2007, Dewerchin and Carmeliet 2012). Inflammatory mediators have a significant influence on the process of angiogenesis (Koch 1998, Ferrara, Gerber et al. 2003), where pro-inflammatory cytokines are known to be potent factors to induce and support angiogenesis (Oura, Bertoncini et al. 2003, Yoo, Bae et al. 2005, Yoo, Yoon et al. 2009) (review (Voronov, Carmi et al. 2014). Of special relevance to angiogenesis, cytokines, in particular TNF-α and IL-1, named as "alarming cytokines" (Voronov, Carmi et al. 2014) significantly contribute to both acute and chronic inflammation resulting in progressive healing (in acute stage) or towards the progressive destruction of tissues and organs (in chronic stage) (Feghali and Wright 1997, Kumar 2012). Additionally, these extremely potent inflammatory molecules with the potential to destroy or heal are also the primary mediators of septic shock (Feghali and Wright 1997). Sepsis is the

leading cause of death in critically ill patients (Angus, Linde-Zwirble et al. 2001) and endothelial dysfunction is a key factor in its pathogenesis (Smadja, Borgel et al. 2012).

Based on previous evidence, Matunga and group hypothesised that vascular endothelium is the principal target of the inflammatory response in sepsis, causing widespread vascular damage/disruption to the endothelium (Mutunga, Fulton et al. 2001). An increase in circulating ECs was observed in patients with sepsis but without shock, suggesting that endothelial damage precedes the clinical development of organ failure (Mutunga, Fulton et al. 2001). The number of circulating ECs in sepsis is positively correlated with the severity of vascular injury (Mutunga, Fulton et al. 2001). Learning about the interactions between cytokines and ECs may, therefore, contribute towards our understanding of the mechanisms involved in endothelial shedding leading to progressive destruction/systemic pathological effects in inflammatory disorders of acute and/or chronic in nature.

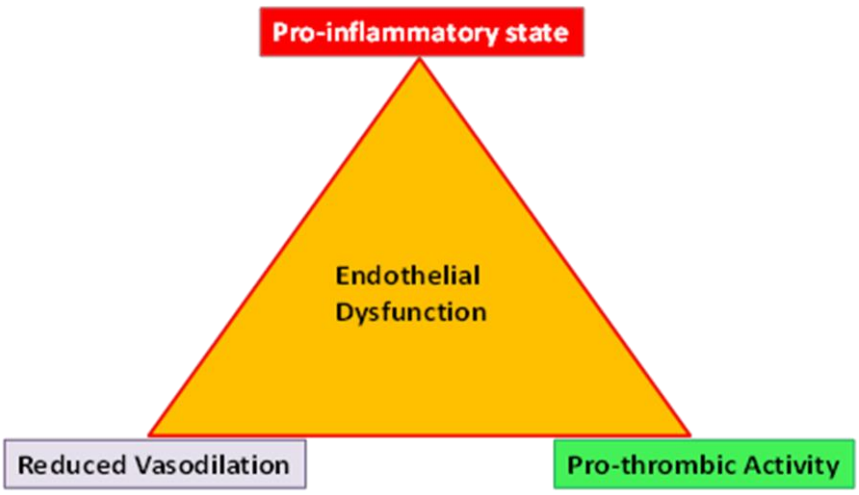


Figure 7.1 Endothelial dysfunction *characterised by a shift in the activities of the endothelium towards reduced vasodilation, a proinflammatory state and prothrombic actions (Rajendran, Rengarajan et al. 2013).*

Vascular endothelium is known to be a specific target of TNF-α (Chia, Qadan et al. 2003, McKellar, McCarey et al. 2009, Zhang, Park et al. 2009). TNF-α signals via its receptor, TNFR1, and induces EC type II acute inflammation. Raised plasma levels of TNF-α are toxic (Lejeune, Lienard et al. 2006) and lead to vascular dysfunction. Effects of TNF-α and inflammatory cytokines are the key mediators of pathophysiology of endothelial/vascular dysfunction associated with several pathologies, including accelerated atherosclerosis, MI, HF (Zhang, Park et al. 2009, Kleinbongard, Heusch et al. 2010, Roubille, Martel-Pelletier et al. 2013) and chronic inflammatory disorders such as RA (Murdaca, Colombo et al. 2012), cancer and PE, and raises risk in trauma patients and obese individuals (Zhang, Park et al. 2009, Roubille, Martel-Pelletier et al. 2013). Among the list of common pathologies (Table 1.6) presenting with higher circulating levels of TNF-α, heart disease (HD) show the highest levels of plasma TNF-α levels ranging between 1000-2000 pg/ml (Lee, Zaske et al. 2011), followed by RA as the second highest. Remarkably, a review by Piero Montorsi states that the atherosclerotic process begins during childhood (Vlachopoulos, Ioakeimidis et al. 2008). Moreover, women with PE and their children are reported to be at a higher risk of developing cardiovascular disease (CVD) at a later stage in life (Smith, Pell et al. 2001, Vatten, Romundstad et al. 2003), wherein TNF-α is reported to play a significant role. Individuals with chronic inflammatory diseases have been associated clinically with excessive cardiovascular risk and increased mortality when compared to the general population (Rosner, Ginzler et al. 1982, Peters, van der Horst-Bruinsma et al. 2004, Gelfand, Neimann et al. 2006, Mathieu, Motreff et al. 2010, Murdaca, Colombo et al. 2012, Nikpour, Gladman et al. 2013, Singh, Singh et al. 2014) (Table 6.1).

Disease	CAD Risk (RR or OR)	Cardiovascular Mortality (RR)
RA	**1.5-2.0** (Solomon, Goodson et al. 2006, Crowson, Liao et al. 2013)	**1.5** (Avina-Zubieta, Choi et al. 2008)
Systemic Lupus Erythematosus	**2.2-2.6** (Hak, Karlson et al. 2009, Magder and Petri 2012)	**1.7** (Bernatsky, Boivin et al. 2006)
Psoriasis (severe)	**1.5-7.1** (Gelfand, Neimann et al. 2006, Samarasekera, Neilson et al. 2013)	**1.1-1.6** (Gelfand, Neimann et al. 2006, Samarasekera, Neilson et al. 2013)
Ankylosing Spondylitis	**1.9** (Peters, Visman et al. 2010, Mathieu, Gossec et al. 2011)	**1.3-2.1** (Mathieu, Motreff et al. 2010)
Inflammatory Bowel Disease	**1.2-1.4** (Fumery, Xiaocang et al. 2014, Singh, Singh et al. 2014)	**1.0** (Dorn and Sandler 2007, Fumery, Xiaocang et al. 2014)

Table 7 .1 Chronic inflammatory diseases and the relative risk of cardiovascular morbidity and mortality manifested by these diseases. RR – Relative risk; OR – Odds Ratio (Steyers and Miller 2014).

TNF-α signaling through TNFR2 activates VEGFR2, promoting angiogenesis; this is a hallmark of chronic inflammation (Review) (Pober and Sessa 2007). VEGFA enhances acute inflammatory effects mediated by TNF-α. The exact mechanisms of signal transduction in these responses are not well understood. Pro-inflammatory signaling may involve an NF-κB stimulation mediated by AKT amplification in some cell types. This pathway, however, this has not been observed in ECs (Pober and Sessa 2007). PlGF modulates the inflammatory process by up regulating VEGF (Bottomley, Webb et al. 2000) and VEGFR-1 (Yoo, Yoon et al. 2009) expression, in addition to increasing production of inflammatory cytokines, such as TNF-α in mononuclear cells (Selvaraj, Giri et al. 2003). An increase in inflammatory cells results in propagation of cytokines and chemokines, some of which are angiogenic, such as VEGF and PlGF (Artese, Rubini et al. 2002). The principle role of TNF-α in endothelial

activation/dysfunction seems to rest on a subtle balance between angiogenic and inflammatory mediator response, leading to systemic protective effects in acute inflammation or systemic pathological effects in chronic inflammation (Kumar 2012).

Hand veins, when exposed to TNF-α, IL-1β or endotoxin for an hour, were caused impaired endothelial function when compared to the hand veins of healthy subjects (Bhagat and Vallance 1997). Endothelial dysfunction persisted for up to 48 hours (Bhagat and Vallance 1997). Moreover, anti-TNF-α treatment improves endothelial dysfunction (Cardillo, Schinzari et al. 2006, Gonzalez-Gay, Garcia-Unzueta et al. 2006, Maki-Petaja, Hall et al. 2006). It is, therefore, fundamental to unravel the possible correlations between circulating pro-inflammatory cytokines and the effects of these mediators on the endothelium resulting in inflammatory angiogenesis, unbalanced homeostasis, and major vascular dysfunction, leading to progressive destruction as a result of chronic inflammatory events.

Although the association between angiogenesis and inflammation is not a new concept, an association of inflammatory mediators, such as TNF-α and endothelial PlGF, which may contribute to an imbalance between pro- and anti-angiogenic factors is poorly understood. In this chapter, the effect of TNF-α on endothelial PlGF and on *in vitro* angiogenesis has been examined.

We believe that this evidence further supports the hypothesis that a conjoined target of angiogenesis and inflammation would reap more beneficial effect in developing therapies inhibiting endothelial dysfunction leading to cardiovascular disease / systemic vascular events.

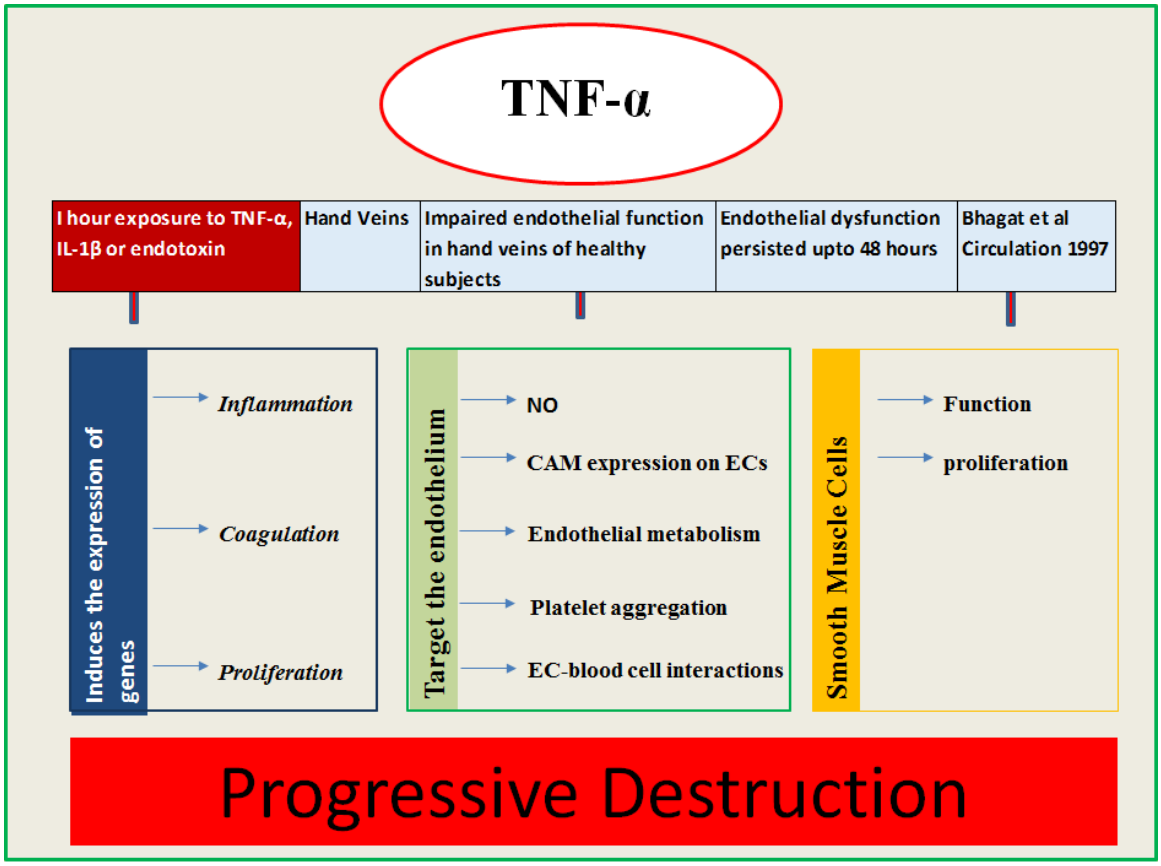


Figure 7.2 TNF-α - the key mediator of endothelial dysfunction and progressive destruction in chronic inflammation. *Inflammation plays a key role in body defence mechanism against external stimuli, such as pathogens or foreign bodies. However, inappropriate and chronic activation of inflammatory processes is an underlying contributor to many common pathological inflammatory conditions. TNF-α and IL-1 are known to be extremely potent inflammatory molecules and share a common feature to drive acute/chronic inflammatory responses. TNF-α secreted by various cells, induces the expression of genes associated with inflammation, coagulation and proliferation, is abnormally increased in the circulation of several disorders and is known to target the endothelium. The vascular effects of TNF-α are alterations in endothelial metabolism/function, platelet aggregation and endothelial cell-blood cell interactions and SMC function and proliferation (Bhagat and Vallance 1997). Abnormally levels of TNF-α drive the angiogenic and inflammatory response in several disorders (Table 1.7). Systemic vascular dysfunction (SVD) could be the minimum common denominator of the several such chronic inflammatory diseases, all of which are associated with high circulating levels of TNF-α, leading to progressive destruction.*

7.2 Results

7.2.1 Endothelial PlGF protein release inhibited by TNF-α.

The abnormal vascular remodelling process implies damage and dysfunction of ECs as well as smooth muscle cell (SMC) proliferation. (Dubey, Jackson et al. 1997, Dubeyl, Rosselli et al. 2000). To investigate whether the effect of TNF-α on PlGF release differs in different cell types, HUVEC, HMEC-1 and SMCs were treated with TNF-α (10 ng/ml) for 24 hours. Cell supernatants were analysed for the levels of PlGF. PlGF protein levels in the presence of TNF-α in both the endothelial cell types, HUVEC and HMEC-1, were decreased by about two-to-three fold compared to the control (Figure 7.3 A, B). Surprisingly, TNF-α had an opposite effect on SMCs and exhibited a three-fold increase in the levels of PlGF compared to the control (Figure 7.3 C). This is the first study to establish a direct effect of TNF-α on the levels of PlGF in cultured ECs.

As TNF-α caused a similar significant reduction in the levels of PlGF release compared to the control in both the EC types [HUVEC, HMEC-1], we chose primary cultures cells, HUVEC for functional studies and HMEC-1 for signalling studies to maximise their respective benefits in their respective experimental boundaries.

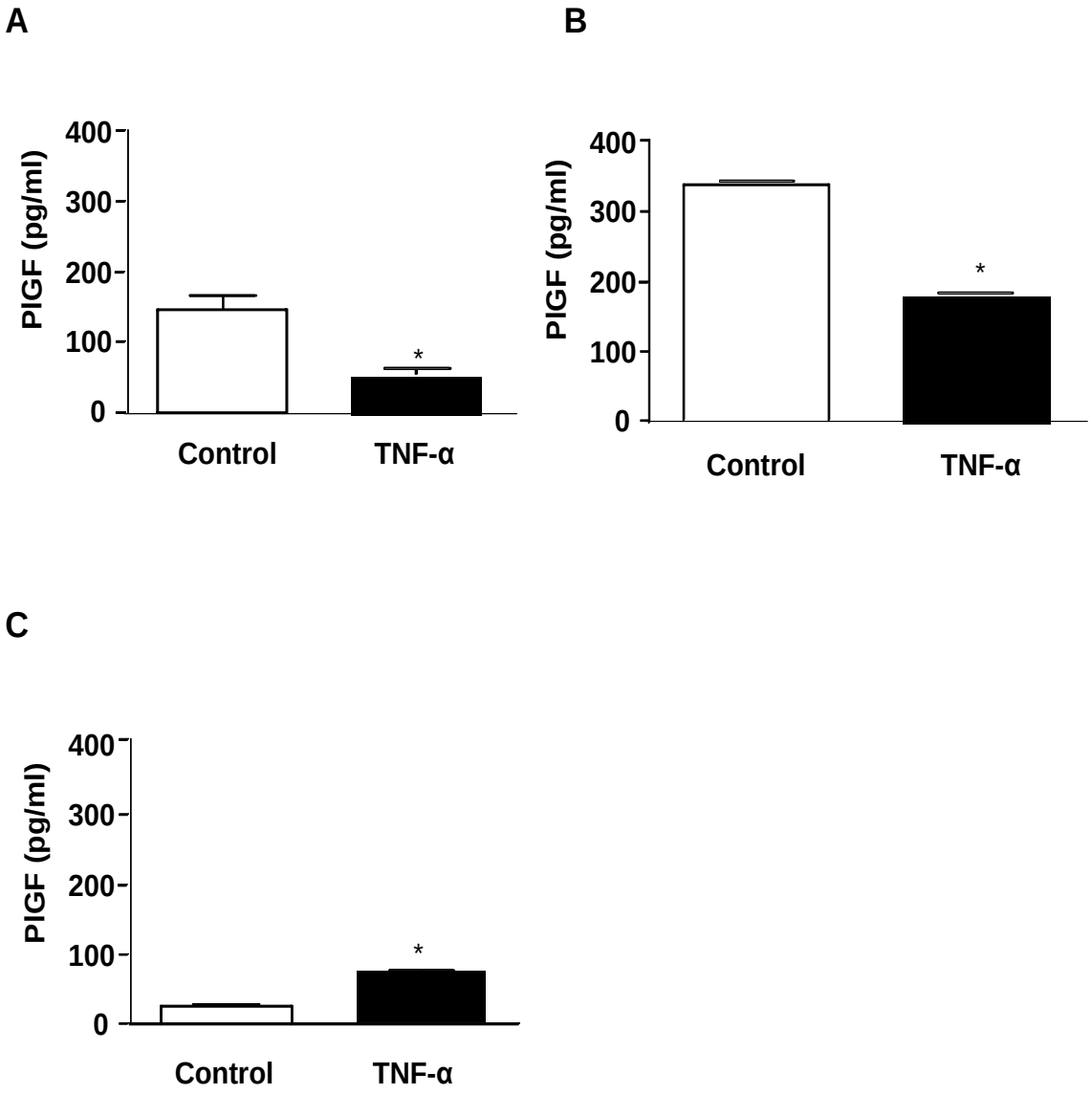


Figure 7.3 The effect of TNF-α on PlGF secretion in different cell types. *Cells were cultured in 24 well plate, and incubated overnight with RPMI medium containing 5% FBS. Cells were stimulated with 10 ng/ml of TNF-α for 24 hours. Cell supernatants from (A) HUVEC, (B) HMEC-1 and (C) SMCs were collected and assayed for PlGF by ELISA. The data represents the mean [± SEM] of 3 independent experiments. [*] P<0.05, [**] P<0.005, [***] P<0.0001 compared to the control.*

7.2.2 PlGF release in response to TNF-α is concentration dependent

To investigate whether TNF-α mediated PlGF release was concentration-dependent, HUVEC were treated with increasing concentrations of TNF-α for 24 hours. A concentration-dependent decrease in the levels of PlGF release in cell supernatants was observed in response to the increasing concentrations of TNF-α. PlGF secretion reached statistical significance from 10 or 100 ng/ml of TNF-α compared to the untreated cells. Treating HUVEC with 10 ng/ml TNF-α caused a significant (two-fold) decrease in PlGF levels (Figure 7.4A). TNF- α concentration of 10 ng/ml was the chosen concentration for the subsequent experiments in this study unless mentioned otherwise. General observation of cell cultures and cell morphology under the microscope of the cells treated with TNF- α displayed no significant differences when compared to the untreated cells.

It was imperative to know if TNF-α effect on PlGF levels is due to the regulatory mechanisms or cytotoxic effects of TNF- α on the cells. To elucidate this, cell viability test in the form of an MTT assay was performed (Figure 7.4B). Cells were subjected to identical culture conditions with increasing concentrations of TNF- α (0.1,1,10,100 ng/ml) as in the above experiment. HUVEC were not subjected to cytotoxicity effects in the presence of varying concentrations of TNF-α when compared to untreated cells.

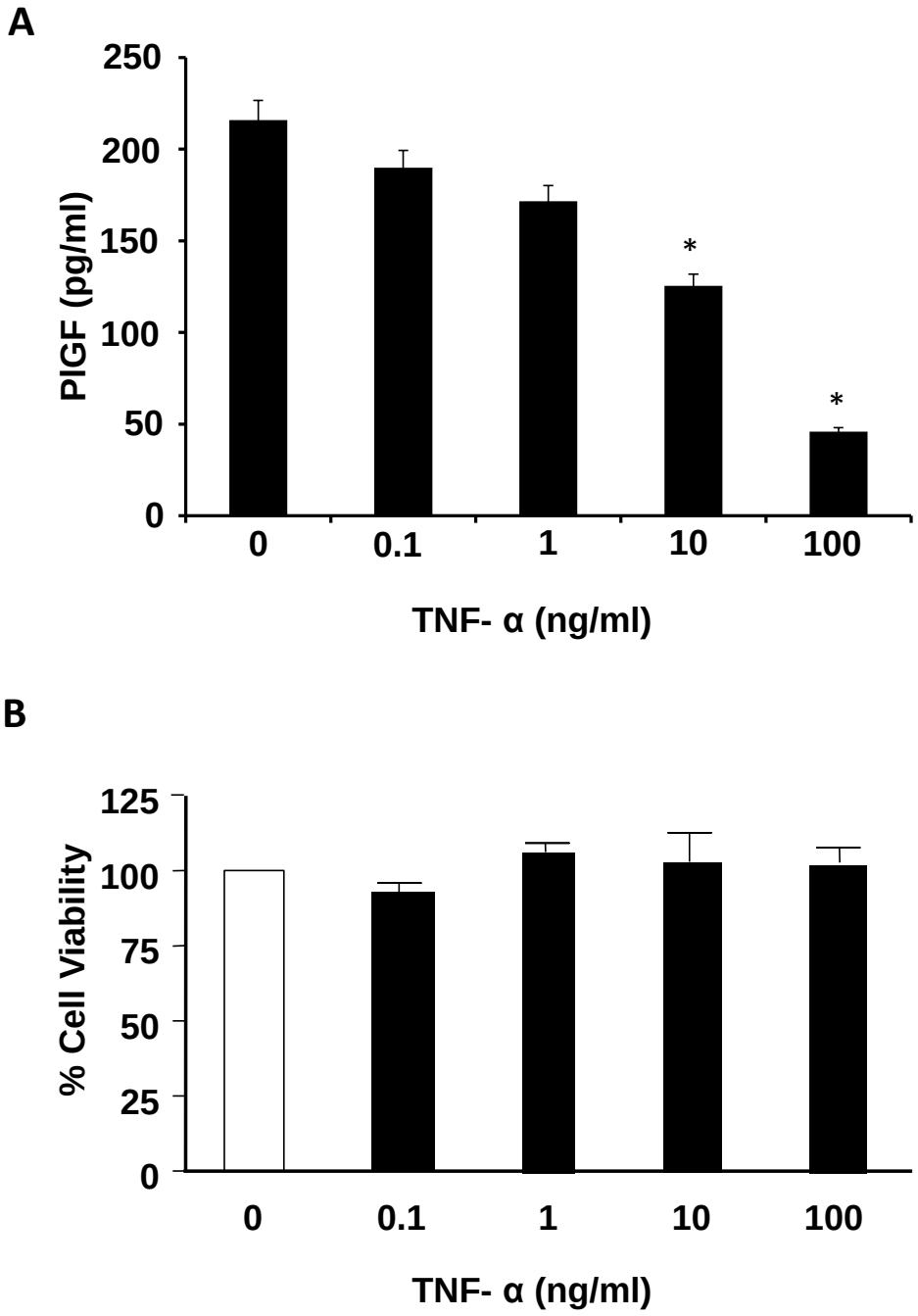


Figure 7.4 TNF-α reduced PlGF release in a concentration-dependent manner. *(A) HUVEC were cultured in serum-reduced medium and incubated overnight. Cells were stimulated with increasing concentrations (0,1,1,10, 100 ng/ml) of TNF-α for 24 hours. The conditioned media was collected and assayed for PlGF by ELISA. (B) Cell viability not effected by TNF-α. HUVEC were seeded in well in a 96 well plate and incubated overnight prior to stimulation. Cells were stimulated and incubated for 24 hours with various concentrations of TNF-α in serum reduced medium. Cell viability was assessed by MTT assay. The data represents the mean (± SEM) of at least 4 independent experiments, performed in duplicate. * p<0.05 vs. Control.*

7.2.3 TNF-α reduces PlGF expression in ECs in a time-dependent manner

To investigate the kinetics of the TNF-α mediated PlGF down-regulation, HUVEC were treated with TNF-α and cell supernatants were collected at various time points and PlGF protein levels assayed by ELISA. Variations in the levels of PlGF release between TNF-α treated and untreated cells became apparent after four hours, and were found to be significant (two-fold decrease) at 8 hours (Figure 7.5 A). PlGF protein levels were noticed to reach its maximum at 24 hours. The effect of TNF-α on PlGF mRNA expression in ECs was determined by real-time PCR performed on cDNA from HUVEC treated with TNF-α for various time periods. Consistent with the protein data, PlGF mRNA expression in the presence of TNF-α was significantly inhibited (Figure 7.5 B). TNF-α induced a two-fold decrease in PlGF mRNA levels at eight hours compared to its control. A decrease in mRNA levels was observed at 16 and 24 hours in TNF-α treated and respective control group as well. To confirm that the TNF-α inhibitory effect on PlGF levels was transcriptionally regulated, cells were incubated HUVEC with actinomycin D, a transcription inhibitor (Sobell 1985), for three or six hours in the presence of TNF-α. The data together demonstrated abrogated levels of PlGF (Figure 7.5 C), confirming the requirement of transcription for PlGF release from ECs.

Collectively, these results suggest that TNF-α reduced PlGF release from HUVEC could be due to reduced *de novo* protein synthesis Twenty four hours was taken as an optimal time for subsequent experiments unless specified.

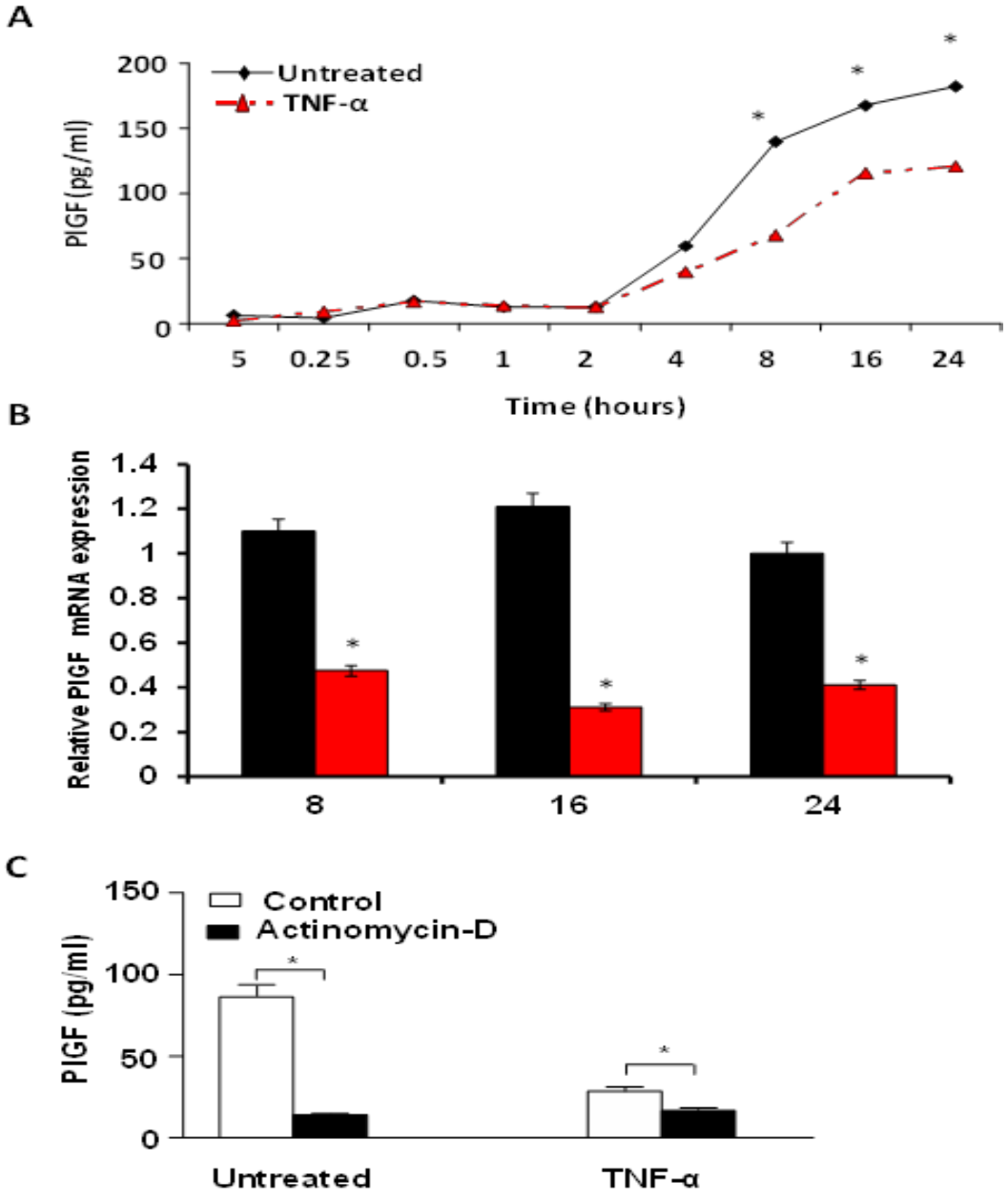


Figure 7.5 Kinetics of TNF-α induced inhibition of PlGF release in HUVECs. *[A] Time-dependent decrease in PlGF secretion in response to TNF-α [10 ng/ml] stimulation. Cells were seeded in a 24 well plate, incubated overnight and stimulated with TNF-α. Cell supernatants were collected at indicated time points and PlGF levels measured by ELISA. [B] TNF-α induced inhibition of PlGF mRNA expression in a time-dependent manner. Quantitative real-time PCR performed to analyse PlGF mRNA levels in HUVEC at 8, 16 and 24 hour stimulation by TNF-α [10 ng/ml]. PlGF mRNA values were normalized to β-actin gene expression. [C] TNF-α inhibited PlGF release is via de novo protein synthesis. HUVEC were pre-incubated with actinomycin-D [1 μg/ml] and then stimulated with TNF-α [10 ng/ml] for 6 hours. Data represntated are mean [±SEM] of at least three separate experiments performed in duplicate. [*] P<0.05, compared to the control.*

7.2.4 TNF-α partially suppresses growth factor-induced PlGF release from ECs.

In Chapter 6 we established FGF-2-induced PlGF production in a concentration-dependent manner. To further investigate the antagonistic activity of TNF-α on PlGF release, HUVEC were treated with FGF-2 [10 ng/ml], or a range of serum concentrations in the presence of TNF-α (Figure 7.6). FGF-2-induced PlGF protein levels were suppressed in a concentration-dependent manner in response to increasing concentrations of TNF-α, up to 10 ng/ml. Interestingly, 10 ng/ml of TNF-α inhibited FGF-2-induced PlGF levels (Figure 7.6A). A similar effect of TNF-α was observed on serum-induced PlGF release (Figure 7.6B). These results demonstrate TNF-α as a potent antagonist of growth factor-induced PlGF secretion.

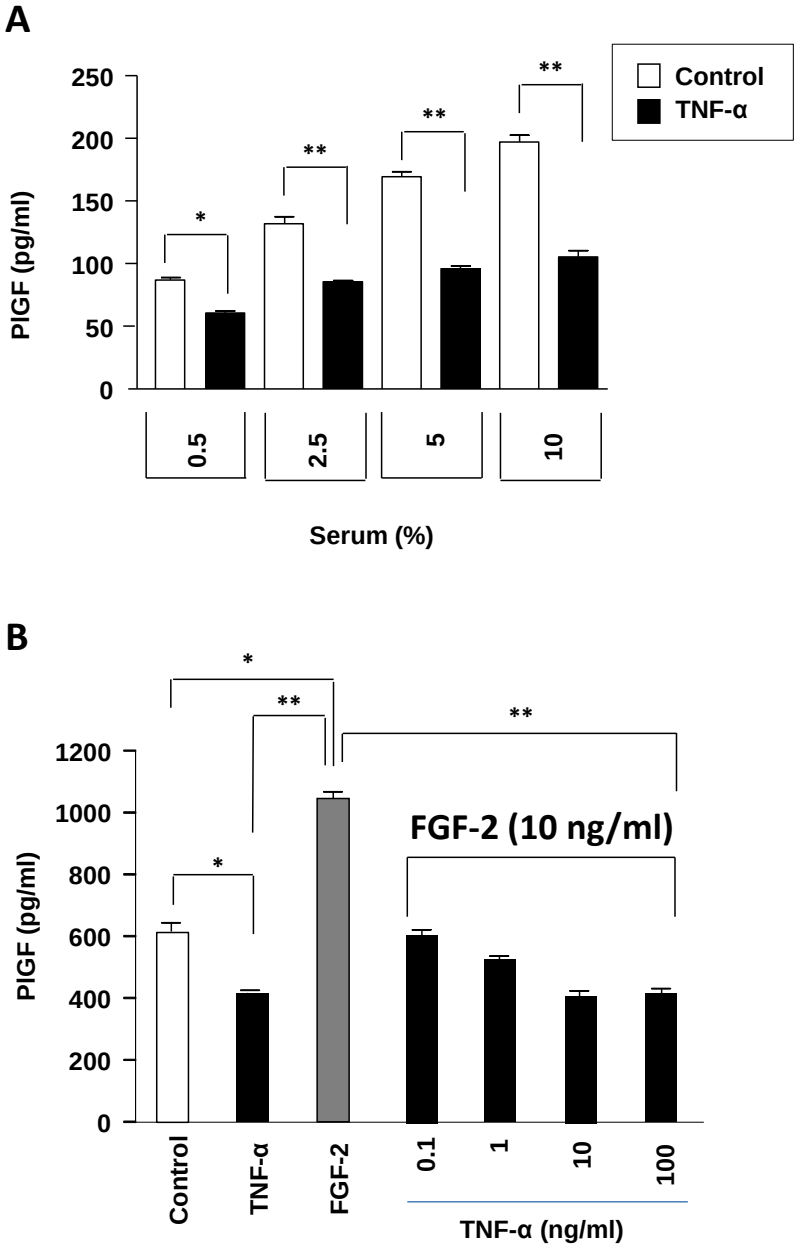


Figure 7.6 TNF-α potentiates the inhibition of FGF-2- or serum-induced PlGF release in ECs. *[A] HUVEC were plated at a density of 1.5x105 per well in 1% gelatin pre-coated 24 well plate. Cells were stimulated with FGF-2 [10 ng/ml] or TNF-α [10 ng/ml] or both with increasing concentrations of TNF-α [0.1, 1, 10, 100 ng/ml] for 24 hours and cell supernatants assayed for PlGF. [B] HUVEC stimulated with increasing concentration of serum in the presence of TNF-α [10 ng/ml], for 24 hours. Cell supernatants collected and assayed for PlGF. Data are mean [±SEM] of three separate experiments performed in duplicate. * P<0.05 compared to Control [UN], ‡ P< 0.005 compared to FGF-2, § P< 0.01 compared to the preceding serum concentration. P<0.0001 between all the groups by one way Anova non-parametric analysis.*

7.2.5 Endothelial cellular functions impaired in TNF- α treated cells

Growth factor and cytokine release induced due to oxidative/sheer stress has been reported by several studies in human and animal models in pathologies, namely tissue ischemia, coronary occlusion and placental insufficiency-intrauterine growth restriction [PI-IUGR] (Regnault, Orbus et al. 2002, Werner, Jandt et al. 2004, Gigante, Morlino et al. 2006). PlGF has been reported to be a significant factor in regulating the angiogenic switch in a variety of pathological states (De Falco, Gigante et al. 2002). Based on the new findings of stress induced PlGF levels in ECs and the possible role of PlGF in endothelial survival mechanisms, we further investigated the effect of TNF-α on PlGF expression and it's effect on cellular functions in ECs.

7.2.5.1 TNF-α inhibits in vitro tube formation.

To further investigate whether TNF-α inhibition on FGF-2-induced PlGF protein release limits angiogenesis, *in vitro* tube formation was studied in HUVEC. Cells were plated on growth factor-reduced Matrigel, in the presence of TNF-α or FGF-2 or FGF-2/ TNF-α. Quantitative analysis showed a significant increase in the total tube length, by about two-fold, when cells were stimulated with FGF-2 (Figure 7.7) [176.6 ± 9.867 µm/field, $p<0.001$, n=4]. A significant reduction in the total tube length [74.20 ± 5.171 µm/field, $p<0.003$ n=4] in the presence of TNF-α was observed as compared to the control (Figure7.7). TNF-α blocked 60 % of FGF-2-induced tube formation, and exogenous addition of FGF-2 in the presence of TNF-α restored tube formation by about 75% percent [133.4 ± 13.46 µm/field, $p<0.03$ n=4], compared to the total tube length in the presence of TNF-α (Figure 7.7). A graphical representation (Figure 7.7B) of the tube formation is shown in Figure 7.7A has been illustrated. These studies clearly demonstrated the inhibitory effect of TNF-α on endothelial

cell capillary network, in addition to the restoration of the capillary network by addition of FGF-2.

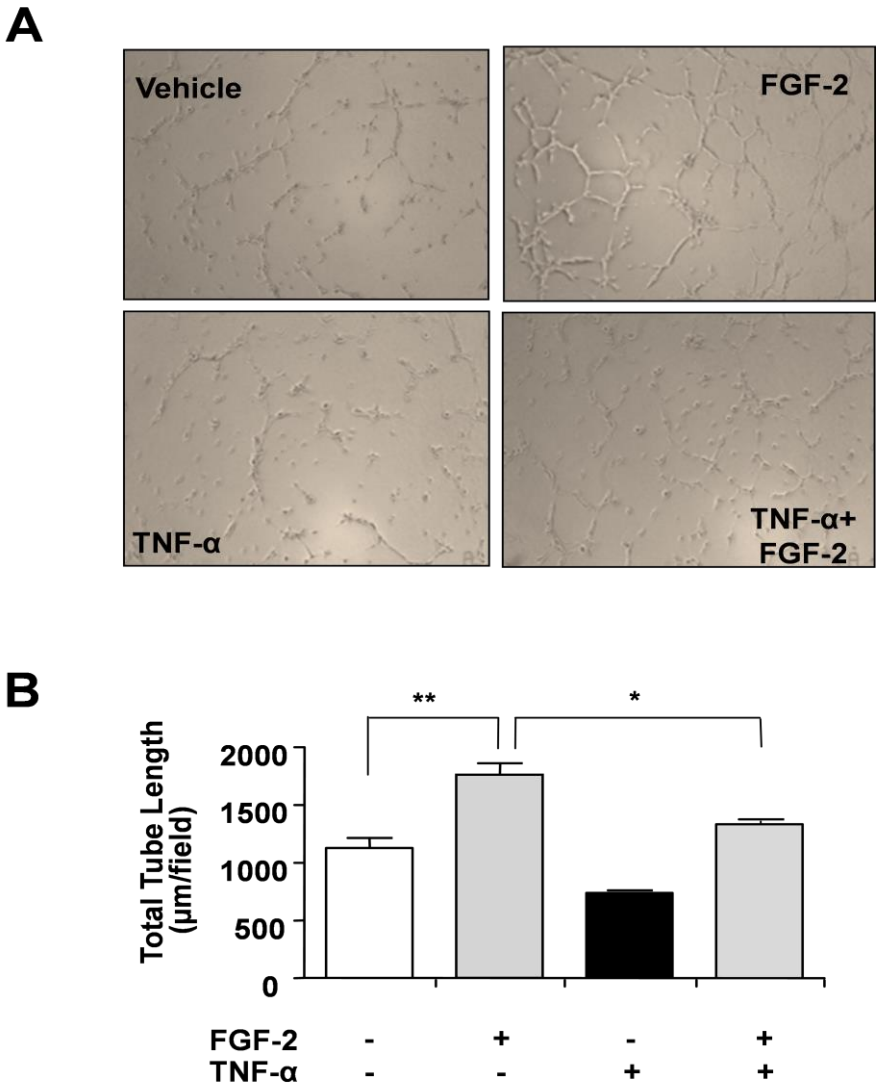


Figure 7.7 FGF-2 restores TNF-α suppressed in vitro tube formation. *[A] HUVEC seeded at a density of 6x104 per well of a 24 well plate, pre-coated with growth-factor reduced matrigel. Cells were stimulated with TNF-α, or FGF-2 or TNF-α/FGF-2 at 10 ng/ml. Cells were observed at six hours after stimulation and results were recorded with a digital system. [B] Graphic representation of quantitative analysis of tube formation in figure 6.7A. Data are expressed as the mean ±SEM of two independent experiments. Statistical comparison was performed using t-test* P>0.01, ** P>0.001 vs. compared to vehicle.*

7.2.5.2 Ability of PlGF to rescue TNF-α inhibited in vitro tube formation.

PlGF restored anti-VEGF-A inhibited capillary morphogenesis in ECs (Kanda, Miyata et al. 2004). We further investigated the role of PlGF to restore the endothelial capillary-like tube network inhibited by TNF-α in ECs. HUVEC were plated on growth factor-reduced Matrigel, in the presence of TNF-α or PlGF or PlGF/TNF-α for 6 hours and capillary-like tube network was analysed. The effect of TNF-α on tube formation at 3 hours showed no significant difference between the control and the treated cells. This was due to the effect of TNF-α on ECs manifested after 4 hours of treatment which was demonstrated in earlier experiments (Figure 7.8 A-B).

Quantitative analysis showed a significant decrease in the total tube length, by about two-fold, when cells were treated with TNF-α (Figure 7.8 A-B) [69.56 ± 3.164, µm/field, $p<0.006$ n=7] compared to the control (Figure 7.8 A-B) [110.7 ± 12.12 µm/field, $p<0.006$, n=7]. Exogenous addition of PlGF, in the presence of TNF-α, restored tube formation completely to 100% percent [114.9 ± 7.100 µm/field, $p<0.7708$ n=7], compared to the total tube length in the untreated group. A graphical representation (Figure 7.8B) of the tube formation in Figure 7.8A has been illustrated. These studies clearly demonstrated the inhibition of tube formation in the presence of TNF-α was completely restored by exogenous addition of PlGF.

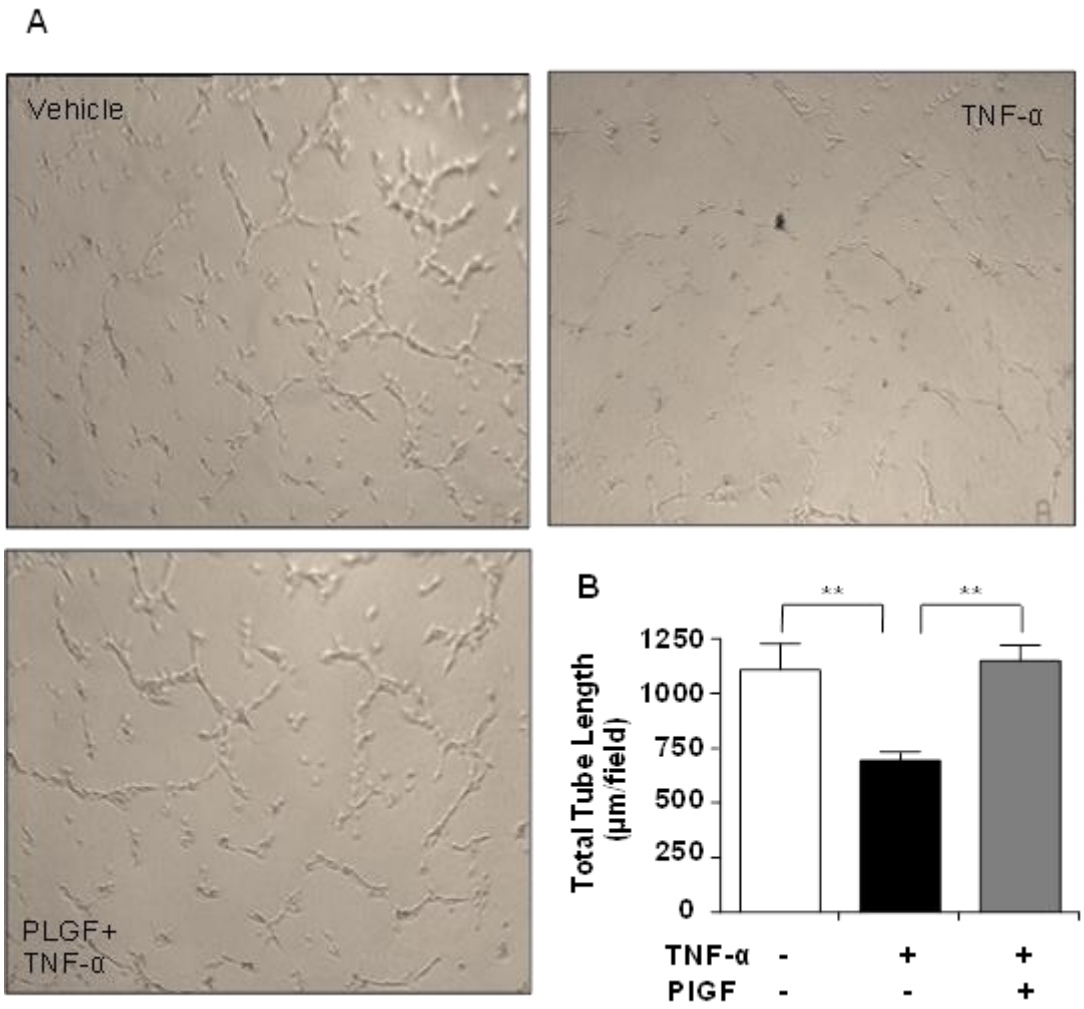


Figure 7.8 PlGF restores TNF-α suppressed in vitro tube formation. *[A] HUVEC were seeded at a density of 6x104 per 24 well plate, pre-coated with growth-factor reduced matrigel. Cells were stimulated with TNF-α, or PlGF or TNF-α/PlGF. Concentration of TNF-α 10 ng/ml, PlGF 50 ng/ml. Cells were observed at 6 hours after stimulation and results were recorded with a digital system.. [B] Graphic representation of quantitative analysis of tube formation in Figure 6.9A. Data are expressed as the mean ±SEM of two independent experiments. Statistical comparison was performed using t-test* P>0.01, ** P>0.001 vs. compared*

7.2.5.3 Endogenous PlGF required for restoration of TNF-α-inhibited in vitro tube formation.

Previous work in our laboratory reported the critical requirement of PlGF for long-term survival of ECs (Cai, Ahmad et al. 2003). We further investigated to know whether the complete restoration of TNF-α inhibited endothelial capillary network required endogenous PlGF, by performing PlGF gene knock down studies. PlGF was silenced in HUVEC by a siRNA approach. The uninfected and infected cells were plated on growth-factor reduced Matrigel in the absence or presence of TNF-α. PlGF silenced cells demonstrated a decrease in the total tube length by about 50 percent in the control group [Figure 7.9]. Wild type as well as PlGF knock-down cells treated with TNF-α had the same levels of total tube length in vitro tube formation decreased by 50 percent (Figure 7.9) when compared to control non-transfected cells. This demonstrated that both TNF-α and PlGF gene inactivation manifest similar levels of inhibitory effects on in vitro tube formation in ECs. Exogenous addition of PlGF in the presence of TNF-α to both uninfected and PlGF silenced cells had a diametrical effect (Figure 7.9). Addition of PlGF to TNF-α treated wild type completely rescued the TNF-α inhibited tube formation, however this was not the case in PlGF silenced cells. Due to the complexity of the experimental readings total tube length measures have not been provided. However, a graphical representation (Figure 7.9B) of the tube formation in figure 7.9A has been illustrated. This clearly ascertains the fundamental requirement of endogenous PlGF for *in vitro* endothelial tube formation.

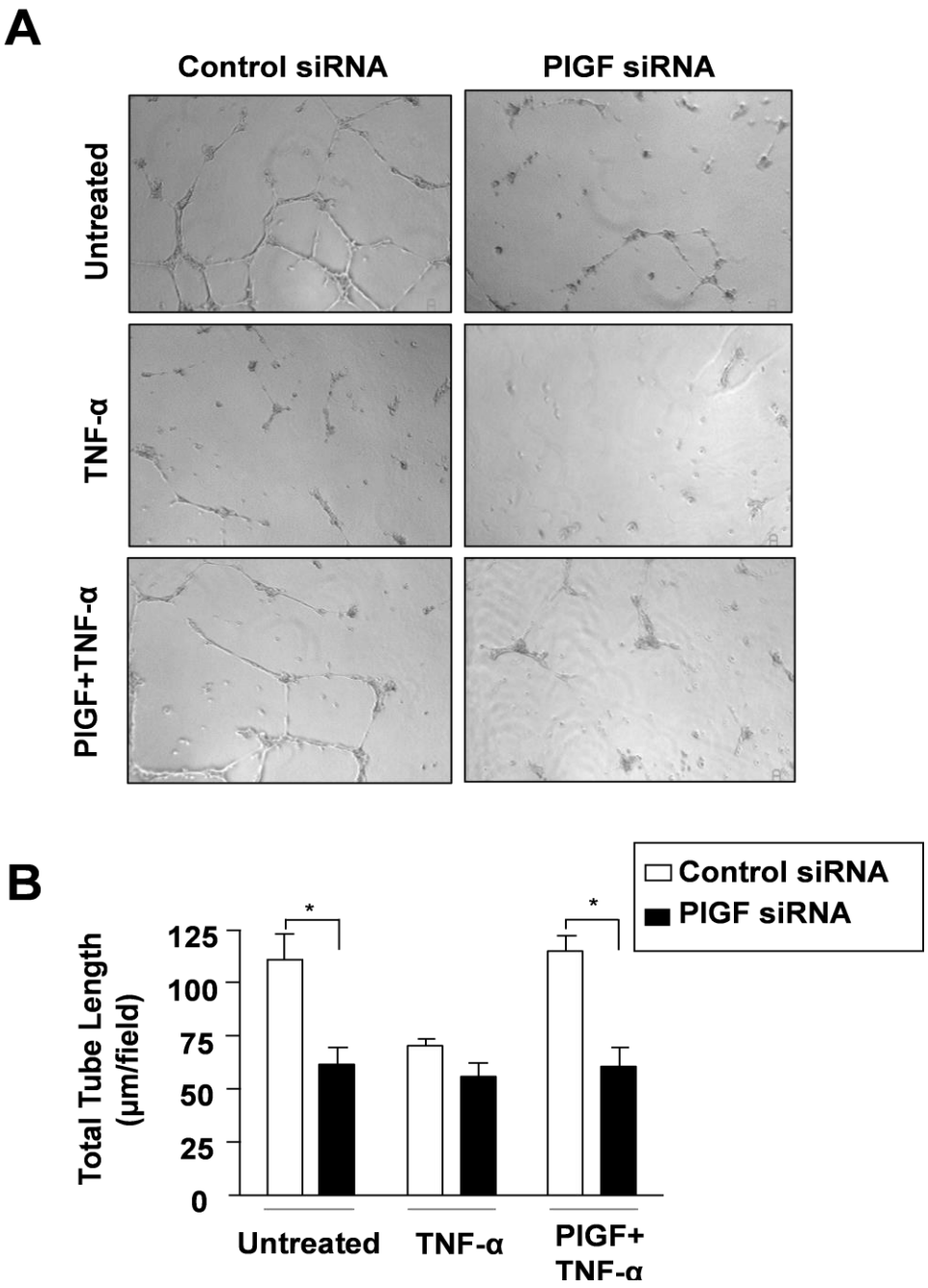


Figure 7.9 PlGF knock-down suppressed in vitro tube formation. *[A] HUVEC seeded at a density of 6x104 per 24 well plate, pre-coated with growth-factor reduced matrigel. Cells transfected by non-specific control [control siRNA] or PlGF siRNA were stimulated with TNF-α, or PlGF or TNF-α/PlGF. Concentration of TNF-α-10 ng/ml, PlGF 50 ng/ml. Cells were observed at six hours after stimulation and results were recorded with a digital system.. [B] Graphic representation of quantitative analysis of tube formation in figure 6.10A. Data are expressed as the mean ±SEM of two independent experiments. Data are expressed as the mean ±SEM of two independent experiments. Statistical comparison was performed using t-test* P>0.05 compared to vehicle.*

7.2.6 PlGF gene inactivation induced apoptosis in ECs.

Apoptosis can be initiated by a wide variety of extrinsic signals such as cytokines, hormones, oxidized lipids or viral agents (Haunstetter and Izumo 1998). The direct effect of PlGF on the survival of tumour ECs and macrophages was mediated by the up-regulation of antiapoptotic gene, survivin, reported in mouse model (Adini, Kornaga et al. 2002). Furthermore, Carmeliet and group demonstrated that murine anti-PlGF monoclonal antibody induced EC apoptosis in tumour models (Fischer, Jonckx et al. 2007). In order to study the role of PlGF in human EC survival, HUVEC were transfected with a control siRNA or PlGF siRNA. Cells were rested for 5-6 hours and were plated at a density of 1 x 10^5 cells per well of a two well microscope chamber slide and incubated overnight. Cells were then treated with 0.2% BSA or 20% FBS for 6 hours and analysed for apotosis. Apoptotic index was measured utilising the TUNEL assay by the detection of apoptotic nuclei, post fixation of the cells. The nuclei of viable cells were stained by DAPI (Figure 7.10A). The TUNEL positive cells incorporate fluorescein-labelled nucleotides into the 30-OH overhangs of DNA fragments, allowing the nuclei to be visualized in a green colour with the aid of a fluorescence microscope, to detect DNA fragmentation at a single-cell level (Figure 7.10A). A significant increase by about two-fold in the percentage of TUNEL positive cells was observed in PlGF gene inactivated cells when compared to control siRNA [$P<0.01$]. Higher concentrations of serum supplemented [20% FBS] to the PlGF inactivated HUVEC could not prevent the occurrence of cell apoptosis, demonstrating the crucial requirement of PlGF for the survival of ECs. The percentage difference in TUNEL positive cells between the 0.2% BSA and 20% FBS treated PlGF gene silenced cells was approximately 10 %. In order to further analyse the conditions that affected PlGF gene knock-down in ECs, experiments were performed in the presence of 20% FBS, to demonstrate the role of PlGF on EC survival at higher serum concentrations.

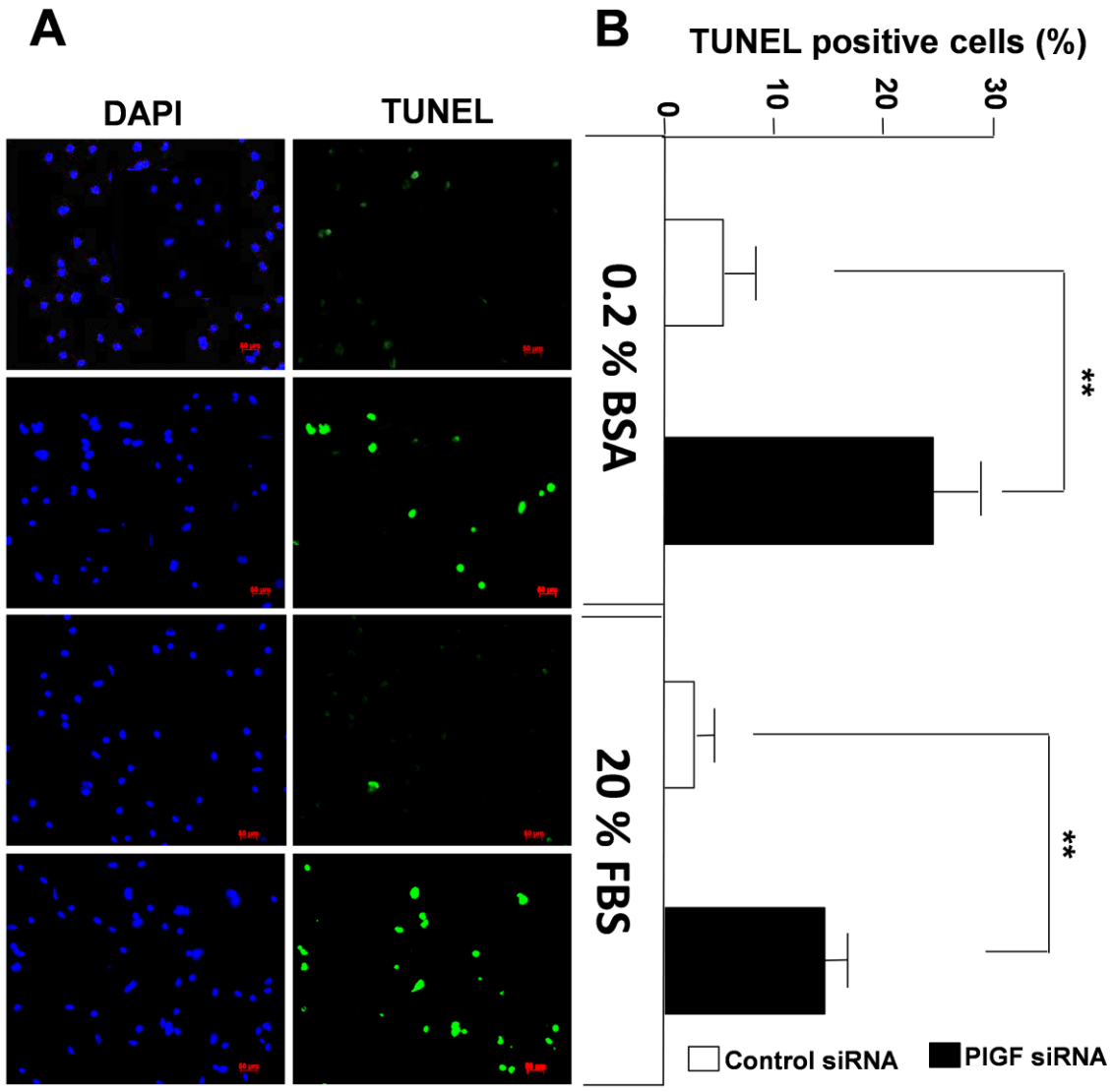


Figure 7.10 Apoptotic index in quantified in PlGF gene silenced cells. *HUVECs were plated at a density of 1x105 on a poly-l-lysine-coated two-well microscope chamber slides treated for 6 hours with 0.2% BSA or 20% FBS. Cells were subjected to the DeadEnd™ Fluorometric TUNEL System involving labelling of DNA fragments at single-cell level with fluorescein-12-dUTP for 60 minutes. Samples were analysed using a fluorescence microscope. [B] Graphical representation of A. *P<0.05 vs. Control siRNA.*

7.2.7 Loss of PlGF induced time dependent EC apoptosis

We further studied the time-dependent apoptotic effect induced by the knock-down of PlGF gene. HUVEC were transfected with a vehicle or PlGF siRNA at a density of 1x 10^5 cells per well of a two well microscope chamber slide with 20% FBS for 6 or 12 hours and analysed for apotosis. A significant increase in the percentage of TUNEL positive cells of about 2 fold at 6 hours and 3 fold at 12 hours of incubation times was observed in PlGF gene inactivated cells when compared to the control [$P<0.01$] (Figure 7.11). A bar graph representation of the experiment result demonstrates the percentage of TUNEL positive cells at both the time points of 6 and 12 hours, in vehicle or PlGF inactivated cells. Although the number of cells positive for TUNEL assay were more in the 12 hour compared to 6 hours in PlGF silenced cells, the difference was not significant. The experiment could not look at subsequent time points, due to the low number of attached cells beyond 12 hours.

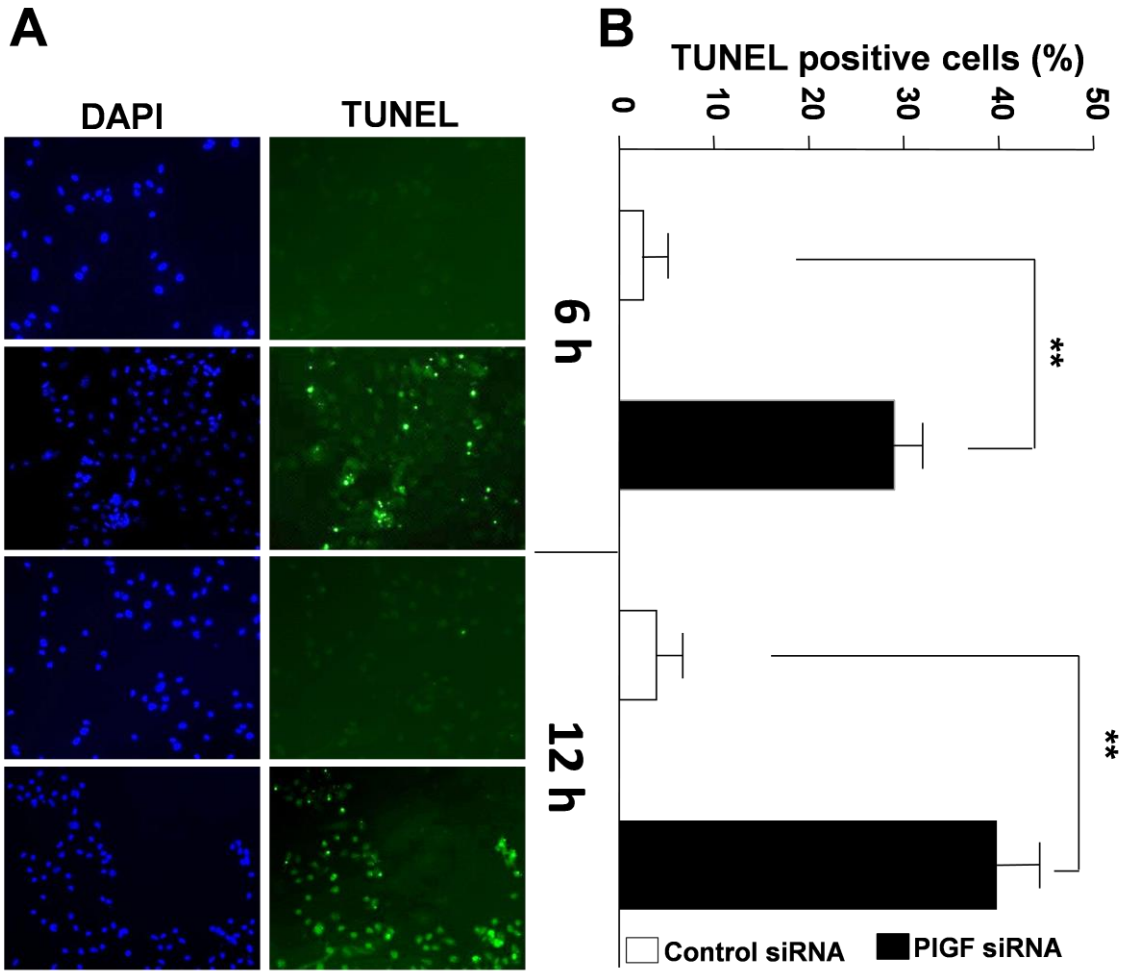


Figure 7.11 Time dependent apoptotic index in PlGF gene silenced cells. *HUVECs were plated at a density of 1x105 on a poly-l-lysine-coated two-well microscope chamber slides treated were incubated for 6 or 12 hours in 20% FBS containing growth medium. Cells were subjected to the DeadEnd™ Fluorometric TUNEL System involving labelling of DNA fragments at single-cell level with fluorescein-12-dUTP for 60 minutes. Samples were analysed using a fluorescence microscope. [B] A graphical representation of Dapi and Tunel positive cells. *P<0.05 vs. Control siRNA.*

7.2.8 Exogenous addition of growth factors does not rescue PlGF gene inactivation induced apoptosis

To determine whether exogenous addition of growth factors reverse effect on the induced apoptosis on PlGF gene inactivated cells, HUVEC were transfected with a control siRNA or PlGF siRNA. PlGF gene inactivated cells were supplemented with PlGF (50 ng/ml) or VEGF (20 ng/ml) or PlGF/VEGF and analysed for apoptosis by TUNEL assay. Interestingly, exogenous addition of these growth factors had no effect on apoptosis induced by the knock down of PlGF gene (Figure 7.12), thus demonstrating that endogenous PlGF critical for EC survival.

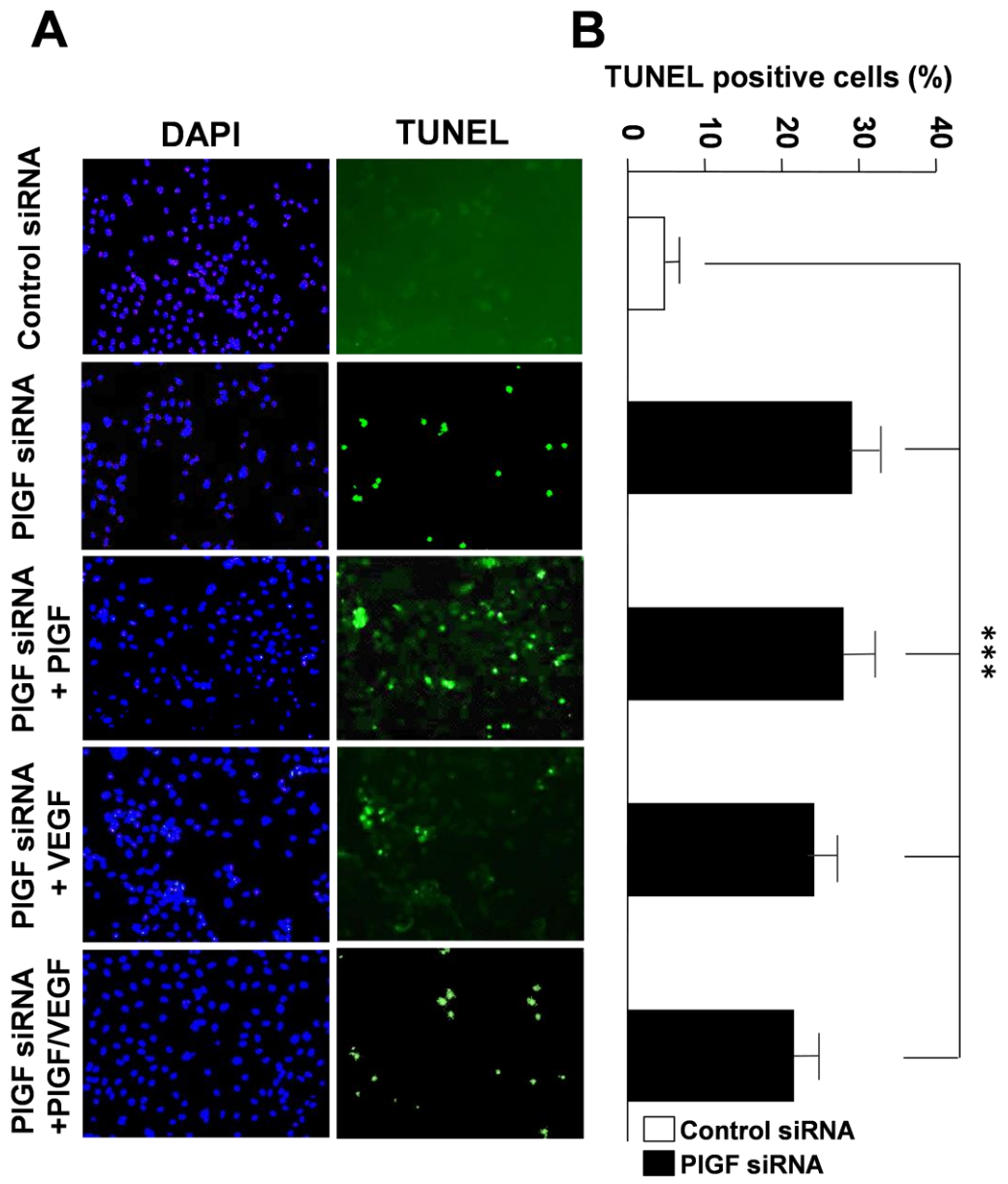


Figure 7.12 Effect of exogenous angiogenic factors on apoptotic index in PlGF gene silenced cells. *HUVECs were plated at a density of 1x105 on a poly-l-lysine-coated two-well microscope chamber slides treated with growth factors; PlGF (50 ng/ml) or VEGF (10 ng/ml) or VEGF+PlGF (10 + 50 ng/ml) for 6 hours in 20% FBS containing cell culture medium. Cells were subjected to the DeadEnd™ Fluorometric TUNEL System involving labelling of DNA fragments at single-cell level with fluorescein-12-dUTP for 60 minutes. Samples were analysed using a fluorescence microscope. [B] Graphical representation of A. *P<0.05 vs. Control siRNA.*

7.2.9 Th1 type Pro-inflammatory cytokines regulate PlGF expression in a concentration-dependent manner in microvascular ECS

To investigate the concentration-dependent effect of the pro-inflammatory cytokines on the levels of PlGF release from Human dermal microvascular endothelial cells (HDMEC), cells were treated with increasing concentrations of TNF-α, IL-1β or INFγ for 24 hours. PlGF was quantified in cell-conditioned medium by ELISA. All three cytokine types significantly suppressed the levels of PlGF compared to untreated cell medium; however, not all the cytokines showed a concentration-dependent effect. TNF-α had no effect on PlGF levels at concentrations up to 1 ng/ml. A concentration-dependent decrease in the PlGF protein levels was observed from 10 ng/ml and higher concentrations (Figure 7.13 A). INFγ significantly suppressed PlGF release at 0.1 ng/ml. Higher concentrations of INFγ [10, 100 ng/ml] had no further effect on PlGF levels (Figure 7.13 B). IL-1β [1, 10 ng/ml] had a dramatic decrease in endothelial PlGF protein release. A concentration-dependent decrease in the levels of PlGF was observed beyond 10 ng/ml of IL-1β (Figure 7.13 C).

TNF-α is a potent Th1-type pro-inflammatory cytokine and has been reported to cause direct damage to ECs, increase EC permeability, up-regulate endothelial adhesion molecules [ICAM-1, VCAM-1, E-Selectin] and promote vasoconstriction, all of which are identified in the pathogenesis of chronic inflammatory disorders, such as PE (Hunt, Chen et al. 1996). In the present study we focused on examining the link between TNF-α on PlGF protein levels in ECs. TNF-α concentration at 10 ng/ml was used as a standard concentration for future experiments unless stated otherwise.

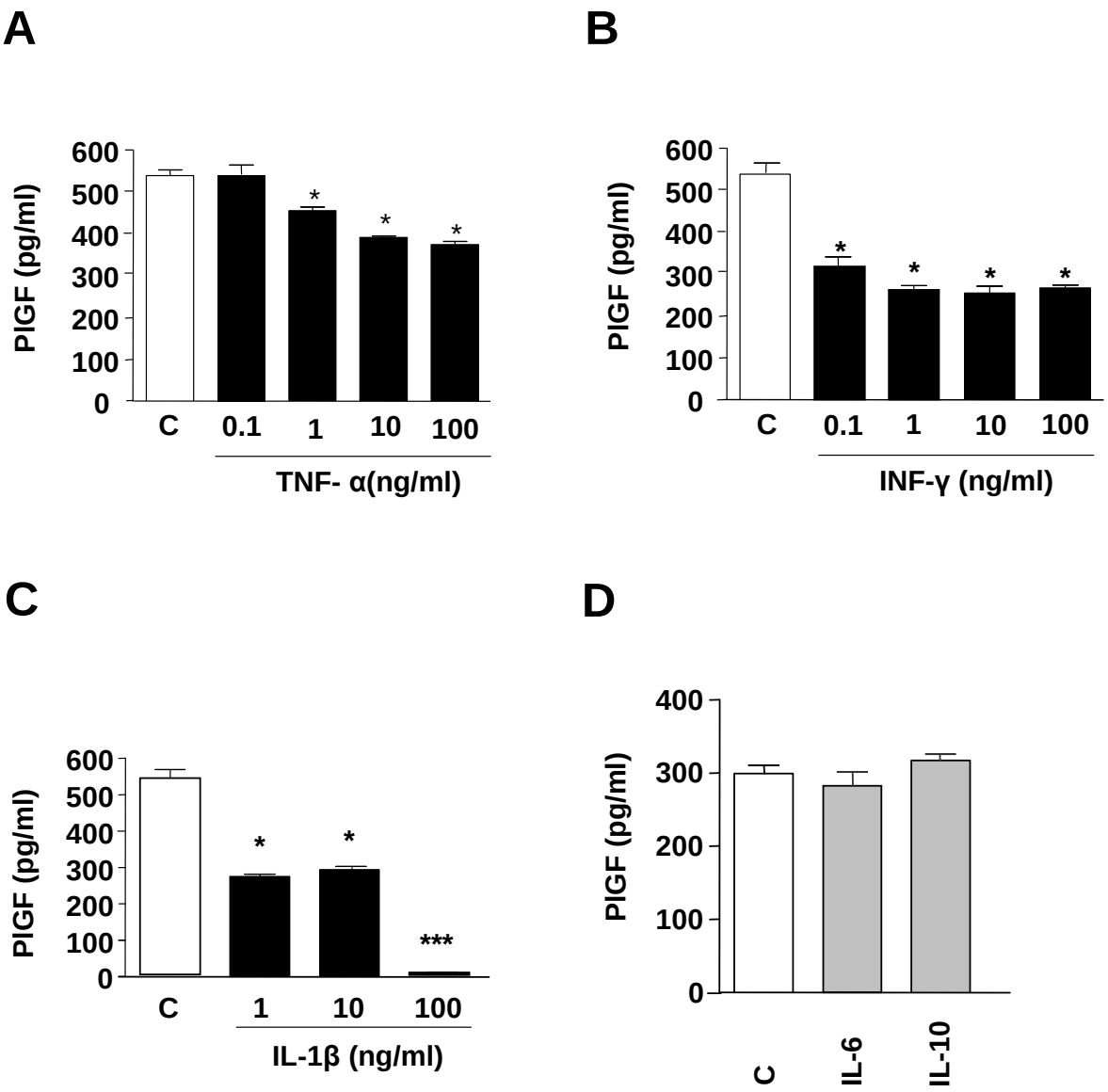


Figure 7.13 Pro-inflammatory cytokines suppress the release of PlGF from ECs. *Confluent. HDMECs were stimulated with increasing concentrations of [A] TNF-α, [B] INFγ, [C] IL-1β or [D] IL-6, IL-10 in 2% FBS containing MCDB131 medium for 24 hours. Conditioned cell supernatants were collected and PlGF levels were measured by ELISA. Data are expressed in pg/ml of free and bound PlGF and are mean [± SEM] of three or more separate experiment performed in triplicates. *P<0.001 vs control [C], ** P< 0.0001 vs C. Part of the data provided by Dr.Samir Sissoui.*

7.2.10 Reduced endothelial PlGF in response to TNF- α in HMEC-1

PlGF protein release from HMEC-1 showed similar response to TNF- α as by HUVEC (Figure 7.3) and HDMEC (Figure 7.13A). Detailed investigation on HMEC-1 to study the kinetics of TNF-α mediated PlGF down-regulation was done.

HMEC-1 were treated with TNF-α, cell supernatants were collected at various time points and PlGF protein levels assayed by ELISA. Variations in the levels of PlGF release between TNF-α treated and untreated cells became apparent after four hours, and were found to be significant (two-fold decrease) at 8 hours (Figure 7.14 A). PlGF protein levels were maximum at 24 hours. The effect of TNF-α on PlGF mRNA expression in ECs was determined by real-time PCR performed on cDNA from HMEC-1 treated with TNF-α for various time periods. Consistent with the protein data, PlGF mRNA expression in the presence of TNF-α was significantly inhibited (Figure 7.14 B). TNF-α induced a two-fold decrease in PlGF mRNA levels at eight hours compared to its control. A decrease in mRNA levels was observed at 16 and 24 hours TNF-α treated and respective control as well. To confirm that the TNF-α inhibitory effect on PlGF levels was transcriptionally regulated, cells were incubated HMEC-1 with actinomycin D, a transcription inhibitor (Sobell 1985) for three or six hours in the presence of TNF-α. The data together demonstrated abrogated levels of PlGF [Figure 7.14], confirming the requirement of transcription for PlGF release from ECs.

Collectively, these results show that TNF-α reduced PlGF release from ECs is due to reduced *de novo* protein synthesis. Twenty four hours was taken as an optimal time for subsequent experiments unless specified.

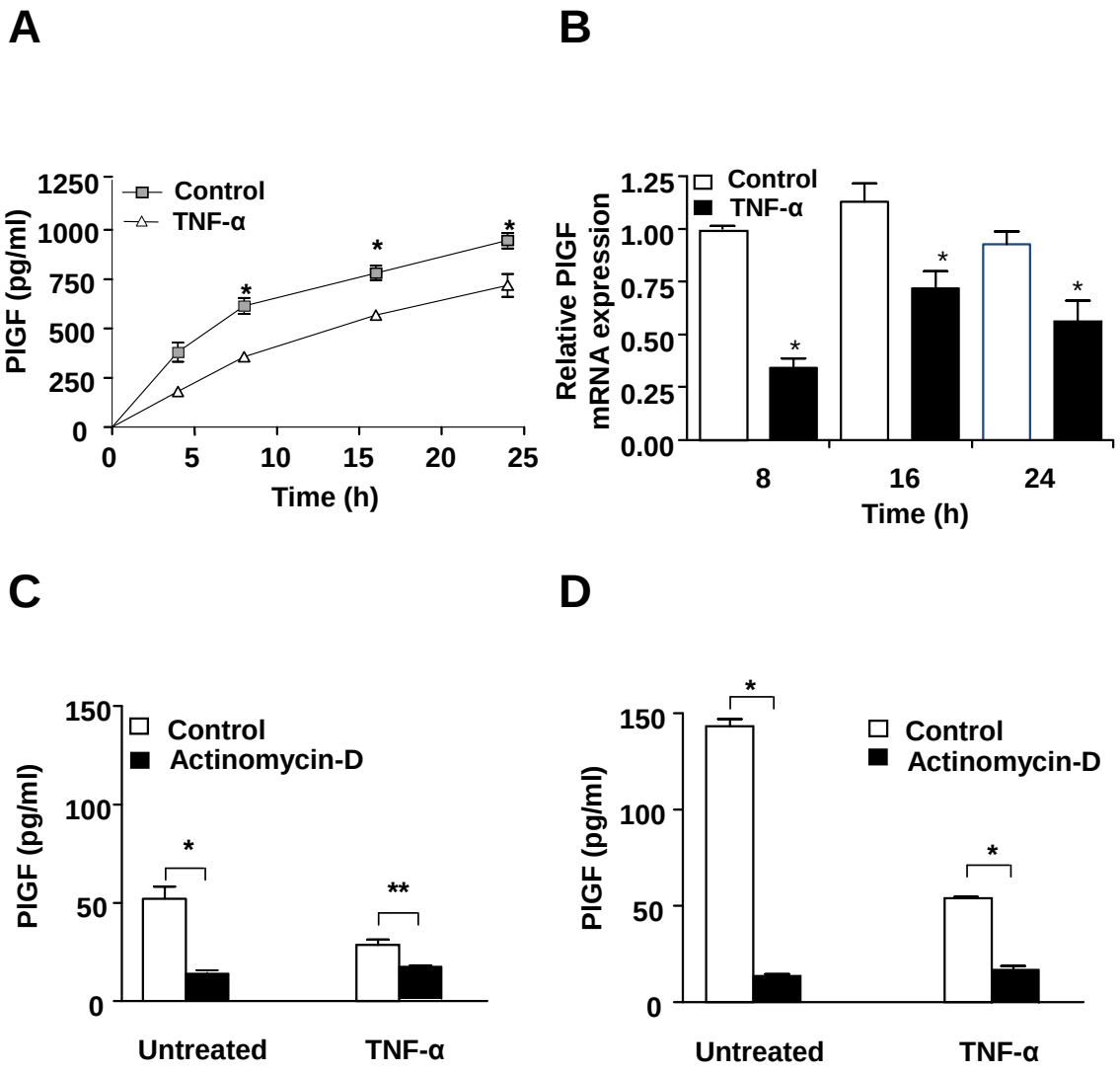


Figure 7.14 TNF-α inhibited PlGF release is transcriptionally regulated. *[A] Time-dependent PlGF protein inhibition from HMEC in response to TNF-[10 ng/ml] stimulation. Cells were seeded at a density of 1.5x105 per well of a 24 well plate, serum starved overnight and stimulated with TNF-α. Cell supernatants were collected at indicated time points and PlGF levels measured by ELISA. [B] TNF-α induced inhibition of PlGF mRNA expression in a time-dependent manner. Quantitative real-time PCR performed to analyse PlGF mRNA levels in HMEC at 8, 16 and 24 hour stimulation by TNF-α [10 ng/ml]. PlGF mRNA values were normalized to β-actin gene expression. [C-D] TNF-α inhibited PlGF release is via de novo protein synthesis. HMEC were pre-incubation with actinomycin-D [1 µg/ml] and then stimulated with TNF-α [10 ng/ml] for 3 [C] or 6 [D] hours. Data are mean [±SEM] of at least three separate experiments performed in duplicate. [*] P<0.05, compared to the control.*

7.2.11 Effect of inflammatory cytokines on PlGF promoter activity in ECs

To study if the reduced PlGF mRNA expression was due to the loss of PlGF promoter activity, 839 bp of the proximal PlGF promoter region was subcloned into pGL3-basic vector [pPlGF*luc*], a luciferase reporter gene. HMEC-1 were transiently transfected with pGL3-basic or with pPlGF*luc* and incubated with TNF-α, IL-1β or IL-6 for 48 hours. The level of promoter activity was measured by dual luciferase assay. We have shown earlier that the Th2 type cytokine IL-6 had no effect on PlGF secretion by ECs (Figure 7.15). Pro-inflammatory cytokines TNF- α and IL-1β modulated PlGF promoter activity. A decrease by about 40% in the activity of PlGF promoter was observed in the presence of TNF-α or IL-1β (Figure 7.15). On the other hand, consistent to the effect of IL-6 on PlGF protein levels, IL-6 had no effect on PlGF promoter activity Additionally, IL-6 acted as a negative control in this experiment. These results were consistent with the reduction in PlGF mRNA and protein levels in the presence of the TNF and IL-1β observed previously (Figure 7.15).

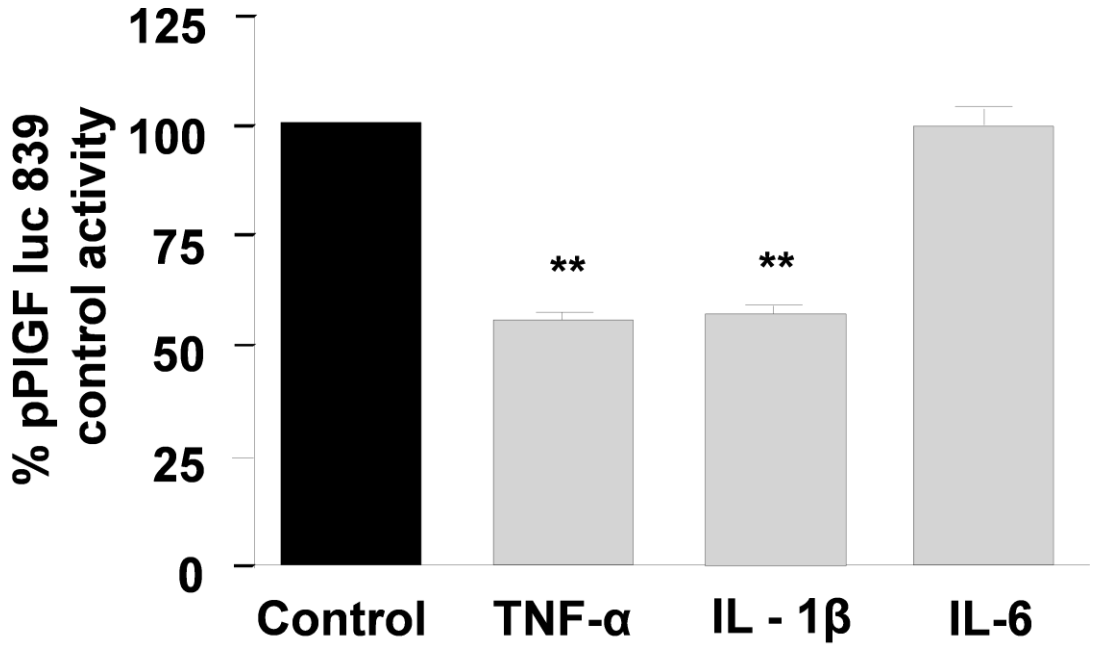


Figure 7.15 PlGF promoter activity in the presence of cytokines. *HMEC-1 were transfected with pPlGFluc [839 bp], on 12 well dish and incubated with 10 ng/ml concentration of the specific cytokines for 48 h. Data are mean [±SEM] of two separate experiments performed in triplicate. [**] P<0.005 compared to the control [untreated]. [Data provided by Dr. Peter Hewett].*

7.2.12 Signalling systems involved in TNF-α-induced PlGF protein reduction in ECs

After confirming the response of HMEC-1 is similar to that of primary cultures in response to TNF-α, a further investigation into the potential signalling enzymes mediating TNF-α-inhibited PlGF release in ECs was undertaken. HMEC-1 were treated with various enzyme inhibitors. As mentioned previously, due to the unlimited proliferation capacity, easy availability and high working passage numbers, HMEC-1 were used for the signalling studies in this chapter.

7.2.12.1 Signaling mechanism involved in TNF-α inhibited PlGF release by ECs

TNF-α activates Src tyrosine kinase and, in response, induces Ca^{2+} influx in HUVEC (Tiruppathi, Naqvi et al. 2001). To investigate whether Src kinase is involved in TNF- α mediated inhibition of PlGF levels in ECs, HMEC were treated with Src kinase inhibitor, PP2. Cell supernatants were analysed for PlGF levels, following a 24 hours incubation. PlGF levels were significantly reduced under basal conditions and in the presence of TNF-α (Figure 7.16). This demonstrates that Src kinase is critical for the production of PlGF from ECs.

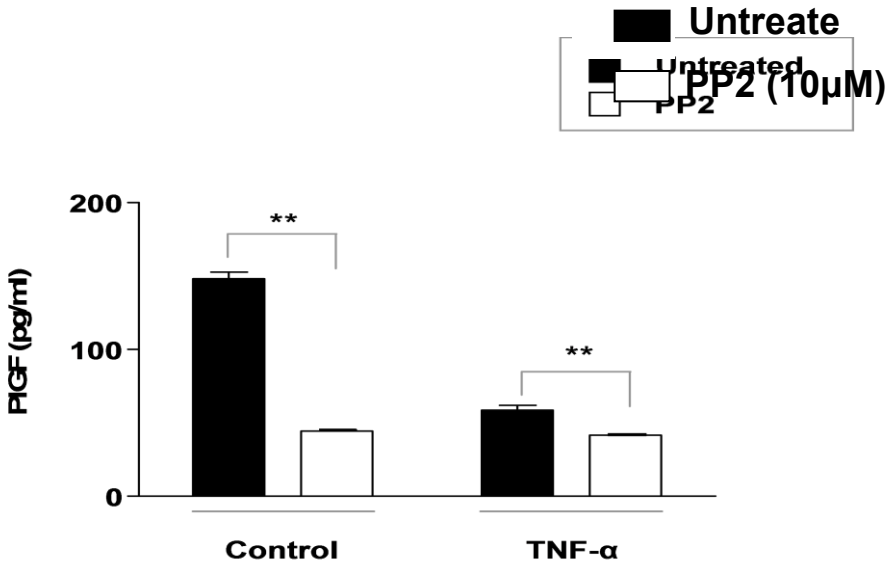


Figure 7.16 Role of Src tyrosine kinase on PlGF release in the presence of TNF-α. *HMEC were pre-incubated with PP2 (10 μM), a specific Src kinase inhibitor for 30 minutes prior to stimulation with TNF-α (10 ng/ml) for 24 hours. Samples were analysed by ELISA for PlGF levels. Data are expressed as means ± SEM of measurements of 4 independent experiments performed in duplicates. **p<0.001 compared to the untreated.*

TNF-α-induced apoptosis (Wang, Liu et al. 2009) and VCAM-1 (Nizamutdinova, Jeong et al. 2008) expression in human vascular endothelial cells (cell line ECV304) and HUVEC respectively via regulation of the PKC pathway. To establish whether PKC signalling is involved in TNF-α-mediated inhibition of PlGF levels, a pharmacological approach to inhibit PKC was used. HMEC were treated with PKC inhibitor, Go6976. Cell supernatants were analysed for PlGF levels, following a 24 hour incubation. PlGF levels were significantly reduced under basal conditions. In contrast, after treatment with Go6976, an increase by 25 percent in the levels of PlGF in the presence of TNF-α was observed (Figure 7.17). This demonstrates that PKC is involved in basal secretion of PlGF from ECs and that Go6976 partially rescued PlGF levels that were decreased in the presence of TNF-α.

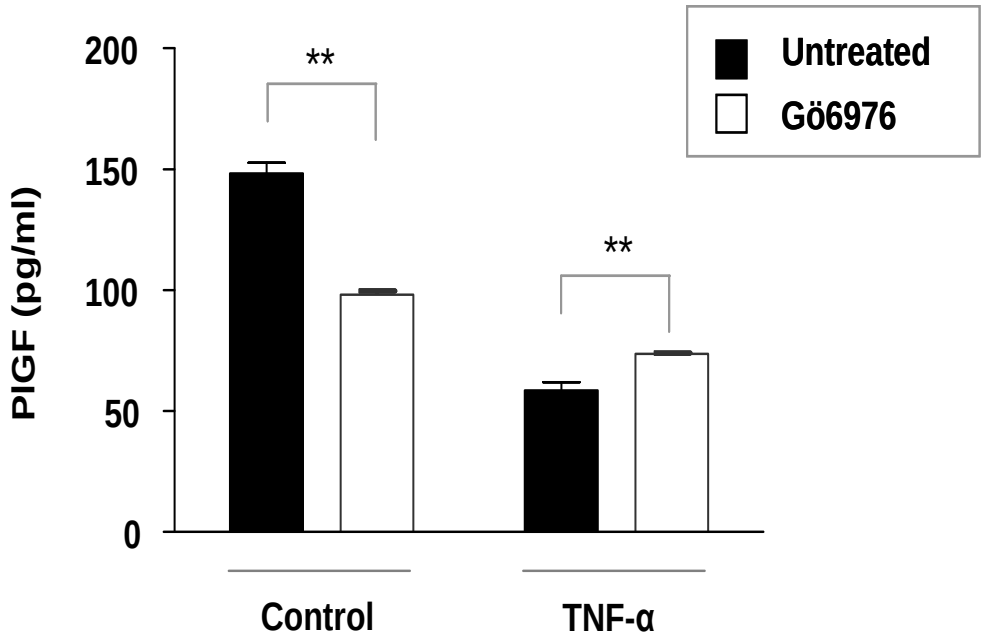


Figure 7.17 PKC inhibitor blocks the inhibition of PlGF release in presence of TNF-α. *HMEC were serum deprived overnight incubated with PKC inhibitor Gö6976 (1 µM) 30 minutes prior to addition of TNF-α (10 ng/ml). Cell supernatants were collected following 24 hour incubation and analysed for PlGF by ELISA. Data are expressed as means ± SEM of measurements of 4 independent experiments performed in duplicate. **p<0.001 compared to the untreated.*

TNF-α-induced down regulation of surface molecules in HUVEC was reported to be independent of MEK and p38 MAPK pathways in HUVEC (Kalsi, Lawson et al. 2002). To investigate whether TNF-α-mediated decreased level of PlGF from ECs followed this mechanism, HMEC were treated with by PD98059, a MEK-1 inhibitor and p38 inhibitor SB 203580. Cell supernatants were analysed for PlGF levels post incubation of 24 hours. PlGF levels were not affected by MEK-1 (Figure 7.18 A) and p38 (Figure 7.18B) inhibitors in the presence of TNF-α, demonstrating that the MAPK kinase pathways are not involved in TNF-α reduced PlGF levels in ECs.

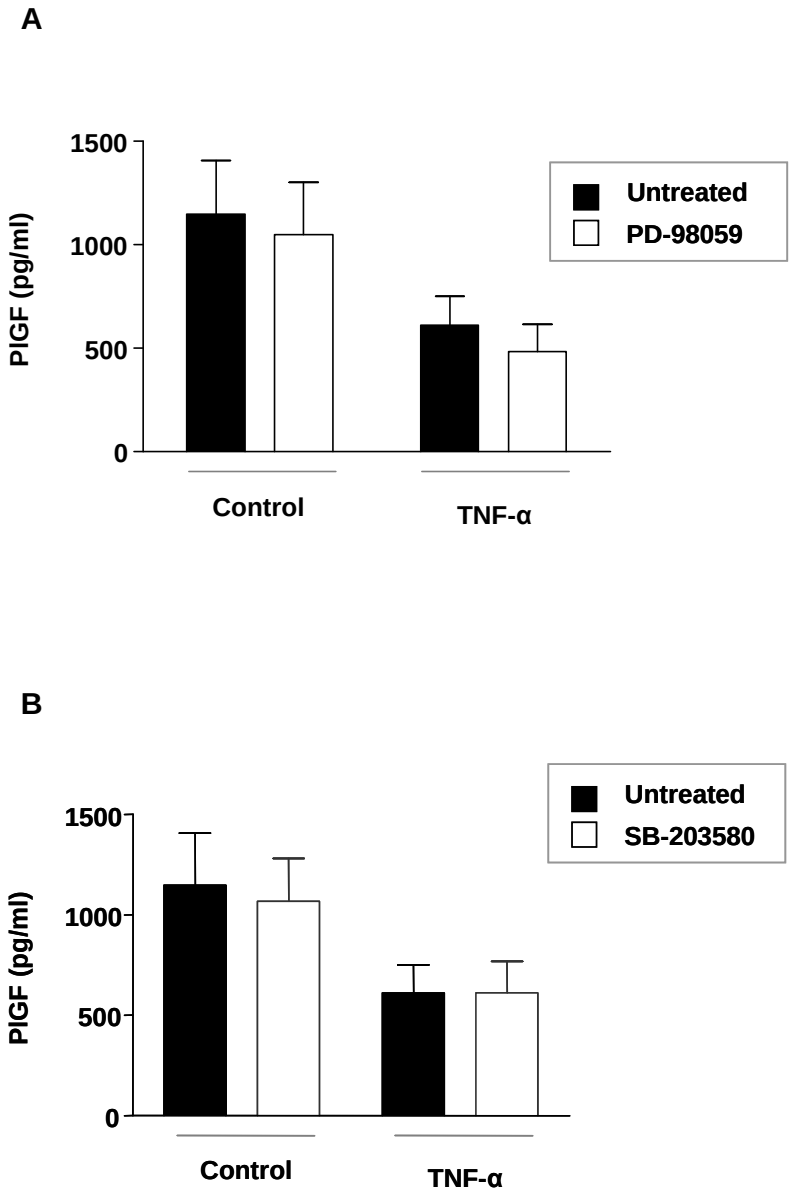


Figure 7.18 MAPK pathway is not involved in TNF-α inhibited PlGF release. *Effect of MEK inhibitor on TNF-α treated PlGF release. HMEC were serum-deprived overnight incubated with MEK1 inhibitor (A) PD98059 (20 µM) or with (B) SB203580 (20 µM), p38 MAP kinase inhibitor, 45 minutes prior to addition of stimulant, TNF-α (10 ng/ml). Cell supernatants collected following 24 hour incubation were analysed for PlGF by ELISA. Data are expressed as means ± SEM of measurements of 4 independent experiments performed in duplicates.*

JNK (Jun N-terminal Kinase), also known as Stress Activated Protein Kinase (SAPK), belongs to the family of MAP kinases (Weston and Davis 2002), and has been reported to be involved in TNF-α activation in ECs (Garin, Abe et al. 2007, Lin, Chen et al. 2007). To confirm whether JNK kinase plays a role in TNF-α-mediated PlGF release, HMEC were treated with JNK inhibitor SP600125. PlGF levels under the basal conditions and in the presence of TNF-α were not affected (Figure 7.19), indicating that JNK kinase is not involved in PlGF release from ECs.

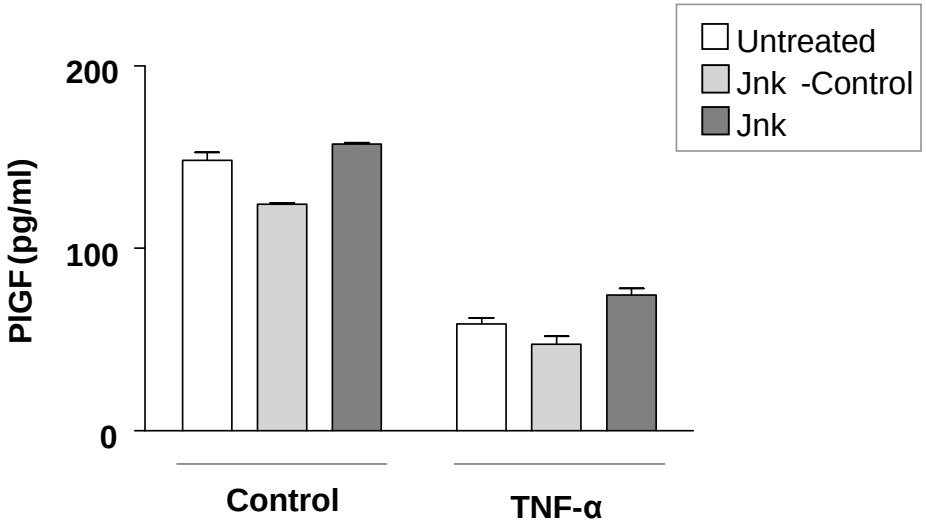


Figure 7.19- JNK kinase does not regulate TNF-α-mediated PlGF release. *HMEC were pre incubated with SP600125 or control inhibitor at 10 µM for 30 minutes, prior to stimulation with TNF-α (10 ng/ml) for 24 hours. Samples were analysed by ELISA for PlGF levels.*

Nuclear factor-kappa B (NF-κB) mobilisation is a well-established transcription factor involved in transcriptional activation of TNF gene (Shames, Selzman et al. 1999). NF-κB is sequestered in the cytoplasm by inhibitory proteins IκBα, and subsequent phosphorylation by a cellular kinase leads to degradation and translocation of NF-κB to the nucleus (Schreiber 1999). NF-κB activation by TNF antagonizes the TNF cytotoxicity (Xu, Bialik et al. 1998, Sumitomo, Tachibana et al. 1999). HMEC were treated with isohelenin, an inhibitor of the pro-inflammatory transcription factor NF-κB (Mazor, Menendez et al. 2000) in the presence of TNF-α and analysed for PlGF levels. Isohelenin induced a significant decrease in the PlGF levels under basal conditions; however, isohelenin had no effect in the presence of TNF-α (Figure 7.20). This indicated that the NF- κB is essential for the release of PlGF from ECs, but the inhibitory effect of TNF-α on the levels of PlGF was independent of NF-κB pathway.

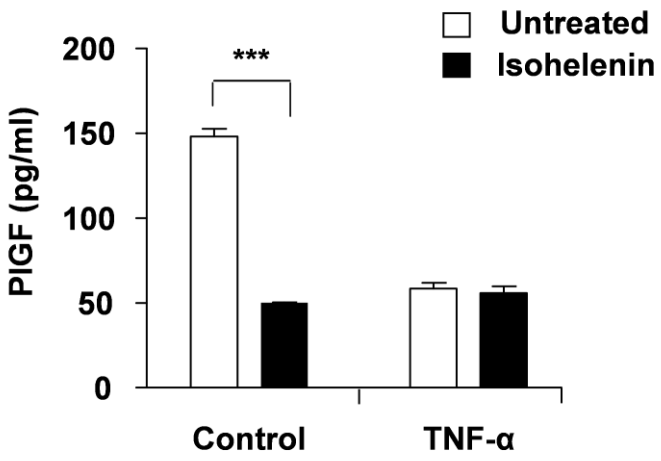


Figure 7.20 TNF-α-mediated PlGF release independent of NF-κB pathway. *HMEC were pre incubated with Isohelenin at 10 µM for 30 minutes, prior to stimulattion with TNF-α (10 ng/ml) for 24 hours. Samples were analysed by ELISA for PlGF levels. ***P<0.0001 compared to the untreated.*

7.3 Discussion

The prominent findings of the present study: 1. we demonstrate for the first time the direct evidence of TNF-α-suppressed endothelial PlGF expression, by combining the results from protein and mRNA analysis, complemented with reduced PlGF promoter activity. 2. TNF-α partially antagonises growth factor-induced endothelial PlGF. 3. TNF-α inhibited tube formation is partially/completely rescued by exogenous angiogenic growth factors. 4. Loss of PlGF function induced EC apoptosis could not be rescued by exogenous angiogenic growth factors. 5. The signalling pathways downstream of TNF/TNFR activation that lead to suppression of PlGF release seem to involve PKC. Our data establish a potential link between TNF-α effect on PlGF in ECs, which perhaps is one of the factors setting the stage for endothelial dysfunction.

In this study, 24 hour exposure of ECs to TNF-α (10 ng/ml) significantly suppressed PlGF levels, compared to the control. This inhibition of PlGF levels was observed in primary micro (HDMEC) and macro (HUVEC) vascular ECs and cell line (HMEC-1). In PE, circulating levels of PlGF are reported to be dramatically reduced (Polliotti, Fry et al. 2003) and it has been associated with inflammation. Increased circulating levels of TNF-α and its receptor (sTNF-rI) are detected in PE and correlate with disease severity (Schipper, Bolte et al. 2005). On the other hand, PlGF deficiency resulted in a diminished and abbreviated inflammatory response (Cianfarani, Zambruno et al. 2006). Modulation of PlGF as a therapeutic target may therefore be beneficial for inflammatory diseases such as psoriasis, RA or tumour angiogenesis (Dubey, Jackson et al. 1997, Bottomley, Webb et al. 2000, Dubeyl, Rosselli et al. 2000, Carmeliet, Moons et al. 2001, Gigante, Morlino et al. 2006, Fischer, Jonckx et al. 2007, Yoo, Yoon et al. 2009).

In contrast to ECs, SMCs had elevated PlGF levels in response to TNF-α. Imbalance between "SMC/EC activators" and "SMC/EC inhibitors" has been suggested to be associated with occlusive disorders such as PE (Dubey, Jackson et al. 1997, Dubeyl, Rosselli et al. 2000). Though our observed findings are potentially limited by several considerations, the results so far are tempting to hypothesise that the differential effect of TNF-α on PlGF levels, endothelial PlGF and SMC secreted PlGF could be a contributing factor associating PlGF to occlusion disorders, such as PE and atherosclerosis, characterised by EC dysfunction/damage, and SMC proliferation/migration (Dubey, Jackson et al. 1997, Dubeyl, Rosselli et al. 2000, Libby, Ridker et al. 2002, Dewerchin and Carmeliet 2012). Moreover, TNF-α concentration is observed to be abnormally high in such diseases, particularly in atherosclerosis when compared to other inflammatory disorders (Lee, Zaske et al. 2011). TNF-α has a central role in EC apoptosis, and is linked to endothelial dysfunction resulting in SVD as a common denominator of several disorders (McKellar, McCarey et al. 2009, Lee, Zaske et al. 2011, Kumar 2012).

We further demonstrate that IL-1β and INFγ also suppress PlGF levels in ECs. Similar to TNF-α, IL-1 (Figure 7.13) plays a distinct role in inflammatory mediated EC dysfunction (Barbieri, Zacchi et al. 2011). TNF-α and IL-1β exposure for even a short period of 1 hour was reported to impair endothelial function in hand veins of healthy subjects (Review) (Huang and Vita 2006). We observed that IL-6 and IL-10 have no effect on endothelial PlGF. IL-10 exerts a protective role (Mallat, Besnard et al. 1999) manifesting its anti-inflammatory effects on the vascular system through inhibition of leukocyte-EC interactions (Sprague and Khalil 2009), in addition to inhibition of pro-inflammatory cytokine and chemokine production by macrophages and lymphocytes as well (Sprague and Khalil 2009). TNF-α and IL-1β, with overlapping properties in the inflammatory process of acute and chronic

inflammation showed a significant reduction in PlGF promoter activity; by contrast IL-6 had no effect what so ever. The effects of these cytokines on PlGF promoter activity were in line with their response on PlGF protein levels secreted by ECs. Further investigation is needed to focus in on the transcription factors regulating PlGF expression and characterization of the specific regions of the promoter responsible for down regulation of PlGF activity, when in the presence of the pro-inflammatory Th1 cytokines. This knowledge would further be transferable to study the causality factors of acute or chronic inflammatory mediated endothelial dysfunction. The present findings so far suggest that pro-inflammatory cytokines significantly suppressed endothelial PlGF secretion in *vitro* and this inhibition is transcriptionally regulated. Henceforth, we believe that this data revolutionises our understanding of inflammatory mediated effects on endothelial disruption to target disease specific angiogenesis.

Although we were greatly tempted to examine further the effect of these inflammatory cytokines on PlGF levels in the interactions between ECs and SMCs, due to practical reasons, such as, limitations in resources and not to detract from our intentions to extensively study endothelial PlGF, we stepped progressively towards TNF-α regulation and its effects on PlGF in ECs alone.

Anti-TNF-α therapy improved high density lipoprotein (HDL) cholesterol levels, as well as micro and macro vascular function in RA patients (Dimitroulas, Sandoo et al. 2012, Sandoo, van Zanten et al. 2012). TNF-α is anti-angiogenic (Sato, Fukuda et al. 1987), perhaps due to its inhibitory effects on eNOS protein expression. TNF-α is reported to suppress eNOS promoter activity and destabilised eNOS mRNA as well (Reviews) (Neumann, Gertzberg et al. 2004, Steyers and Miller 2014). NO reduction is considered to be the hallmark of endothelial dysfunction (Davignon and Ganz 2004, Zhang, Park et al. 2009, Steyers and

Miller 2014), a common factor in several inflammatory disorders. The observed association of functional effect of TNF-α on NO availability and endothelial dysfunction has been established in pre-clinical and human models (Steyers and Miller 2014). NO release in HUVECs in response to PlGF (previous work from our lab) (Bussolati, Dunk et al. 2001), and the up-regulation of eNOS expression by FGF-2 (Mata-Greenwood, Liao et al. 2008), has prompted us to investigate the anti-angiogenic effect of TNF-α in response to these growth factors.

Growth factor-induced PlGF levels were suppressed by TNF-α. TNF-α significantly suppressed serum- or FGF-2-induced PlGF levels, exhibiting the strong antagonist effect of TNF-α towards endothelial PlGF. Interestingly, TNF-α inhibited tube formation was partially rescued by exogenous FGF-2 and completely rescued by exogenous PlGF (Figures 7.7, 7.8). Furthermore, loss of function experiments with PlGF gene silenced ECs subjected to *in vitro* tube formation demonstrated truncated response. PlGF siRNA cells (untreated cells, or treated with TNF-α or TNF-α + PlGF) displayed reduced capillary network that matched to TNF-α inhibited capillary network in non-specific control siRNA group (Figure 7.9). Although TNF-α-inhibited tube formation in the control group ECs (control siRNA) were salvaged when supplemented with exogenous PlGF, understandably PlGF silenced ECs could not be redeemed (Figure 7.9). These findings ascertain the critical role of PlGF for EC tube formation, and that the effect of total rescue manifested by PlGF on TNF-α-attenuated tube formation is a solitary contribution by PlGF alone.

Unlike, VEGF, studies conducted earlier in our laboratory established PlGF sustained angiogenesis and survival of ECs by up-regulation of anti-apoptotic protein bcl-2 expression (Cai, Ahmad et al. 2003). Furthermore, Carmeliet and group demonstrated that murine anti-PlGF monoclonal antibody induced EC apoptosis in tumour models (Fischer, Jonckx et al.

2007). Mice have only one PlGF gene, when compared to humans that have two forms; PlGF-1 and PlGF-2 differ in the ability to bind to neuropilin. Based on previous evidence and our current observations, we hypothesised that the dramatic reduction in capillary network demonstrated by PlGF gene silenced cells perhaps was due to EC apoptosis. Testing our hypothesis by loss of function experiments, TUNEL assay established this to be true. EC apoptosis is reported to be one of the factors contributing to endothelial dysfunction (Asai, Kudej et al. 2000, Tricot, Mallat et al. 2000); a leading cause in several pro- or anti-angiogenic inflammatory disorders (Lee, Zaske et al. 2011, Kumar 2012, Steyers and Miller 2014). PlGF is expressed by migrating keratinocytes and ECs, acting in a paracrine and autocrine fashion on VEGFR-1-expressing endothelium (Failla, Odorisio et al. 2000). Lack of PlGF resulted in delayed wound closure, indicating that it is required for optimal skin repair (Carmeliet, Moons et al. 2001). Over expression of this growth factor accelerated wound closure in diabetic mice (Cianfarani, Zambruno et al. 2006). In the TUNEL assay PlGF gene silenced cells demonstrated an increase in apoptosis rate in low serum conditions and in prolonged incubation time (12 hours). Exogenous supplementation of potent growth factors, such as, PlGF or VEGF or PlGF and VEGF together, did not rescue cell apoptosis in PlGF inactive cells, even when cells were grown in high serum concentration (20% FBS) contained endothelial growth medium. The important finding that potent angiogenic factor VEGF, known for its survival feature being unable to rescue loss of PlGF-induced EC apoptosis, is perhaps due to the combined effects of PlGF being unavailable and the presence of sVEGFR-1, a negative regulator of endothelial function. In 2011, our group made a vital contribution that unlike PlGF, VEGF-A stimulation of HUVEC increased sVEGFR-1 secretory protein and mRNA levels into the culture medium (Ahmad, Hewett et al. 2011). Pre-incubation of HUVEC with VEGFR-2 selective inhibitor SU1498, blocked the VEGF-A induced sVEGFR-

1 release (Ahmad, Hewett et al. 2011). Additionally, recent work by Dr Sissaoui demonstrated an increase in PlGF release in response to VEGFR-1 knockdown suggesting a possible autocrine loop regulating PlGF and VEGFR-1 expression. Inactivation of PlGF gene therefore may have compound effect on endothelial function. It is difficult to explain the distinct contributions of VEGFR-1 and R2 with the current results, highlighting the need to study in detail how these receptors specifically contribute to PlGF induced endothelial apoptosis and subsequently endothelial dysfunction.

Several lines of evidence have demonstrated the critical role of endothelial apoptosis leading to endothelial dysfunction being linked to a growing list of inflammatory disorders (Asai, Kudej et al. 2000, Tricot, Mallat et al. 2000). Tricot and group examined EC apoptosis by terminal dUTP nick end-labelling and ligase assay in the luminal endothelium of 42 human carotid atherosclerotic plaques retrieved by endarterectomy (Tricot, Mallat et al. 2000). A systematic preferential occurrence of apoptosis was observed in the downstream parts of plaques, where low flow and low shear stress prevailed when equated to the upstream parts (Tricot, Mallat et al. 2000). Suggesting that local stress *in vivo* influences EC apoptosis conceivably postulates this as a major determinant of plaque erosion and thrombosis, as well as a possible link between EC apoptosis to human atherosclerosis and its relation to blood flow (Tricot, Mallat et al. 2000). It would have been interesting to measure the levels of PlGF and pro-inflammatory cytokines, in particular, TNF-α. IL-1β and INFγ that suppress endothelial PlGF (present study), to examine if a link persisted between these inflammatory and angiogenic mediators on EC apoptosis and subsequent EC dysfunction in such disorders.

Obstructive sleep apnoea (OSA) has been linked to cardiovascular and cerebrovascular morbidity and mortality (El Solh, Akinnusi et al. 2007). Individuals with OSA have increased

propensity for of 4.6 and 8-fold higher risk for coronary artery disease (Peker, Carlson et al. 2006) and stroke (Palomaki 1991) respectively. Patients with OSA have high numbers of circulating apoptotic ECs correlated to abnormal vascular function (El Solh, Akinnusi et al. 2007). Several factors, including increased oxidative stress with lowered antioxidant capacity, reduced endothelial NO production, activated inflammation, and increased EC apoptosis combine to contribute to unfavourable cardiovascular effects of OSA that may result in endothelial dysfunction and unstable plaque formation (Nakashima, Henmi et al. 2013). Moreover, in children with OSA syndrome (OSAS) an association between endothelial dysfunction and neurophysiological deficits has been established (Gozal, Kheirandish-Gozal et al. 2010), which compels future research to focus in further detail on inflammatory mediated EC apoptosis and endothelial dysfunction.

An attempt was made to delineate the underlying signalling molecules governing TNF-α-suppressed PlGF levels secreted by ECs using pharmacological inhibitors for specific signalling molecules. However, we could not find any known signalling enzymes that could overcome the inhibitory effect of TNF-α on PlGF production. Major signalling molecules such as, NF-κB, Src kinase, PKC, MAPK and JNK were examined in this process.

As illustrated in this study, EC apoptosis induced by loss of PlGF, due direct or indirect effect of TNF-α presence we think perhaps this is the leading cause of endothelial dysfunction. Endothelial dysfunction is a central causality factor for several acute and chronic inflammatory disorders that have raised risk of CVD. Plasma levels of multimeric glycoprotein von Willebrand factor (VWF), produced by ECs (Hollestelle, Thinnes et al. 2001) and a well characterised marker of stroke, vascular events, CVD (Review) (Spiel, Gilbert et al. 2008), are raised in different states of endothelial damage. Blockade of NO

enhances release of VWF in humans and is useful marker of endothelial dysfunction (Review) (Spiel, Gilbert et al. 2008). Study of endothelial dysfunction in PlGF loss of function experiments by analysing VWF on PlGF siRNA EC/SMC functions, such as angiogenic assays, TUNEL or flow cytometry assays *in vitro* and *in vivo* PlGF KO disease models would identify whether the differential effect of TNF-α on PlGF secretion by ECs and/or SMCs that is more robust subsequently results to endothelial dysfunction perhaps the causality factor occlusive disorders, such as atherosclerosis.

In summary, we believe that the current results demonstrate a potential link between TNF-α, IL-1β (cytokines mainly involved in leading to systemic pathological effects in chronic inflammation) and PlGF. Loss of PlGF induced EC apoptosis perhaps may be the causality factor in endothelial dysfunction, a common causality factor of several inflammatory disorders.

The interplay between inflammation and angiogenesis is a mine field. However, we would like to believe that this data would enable and contribute towards our understanding of the several complex interactions between chronic inflammation and angiogenesis aiming to achieve more effective therapies.

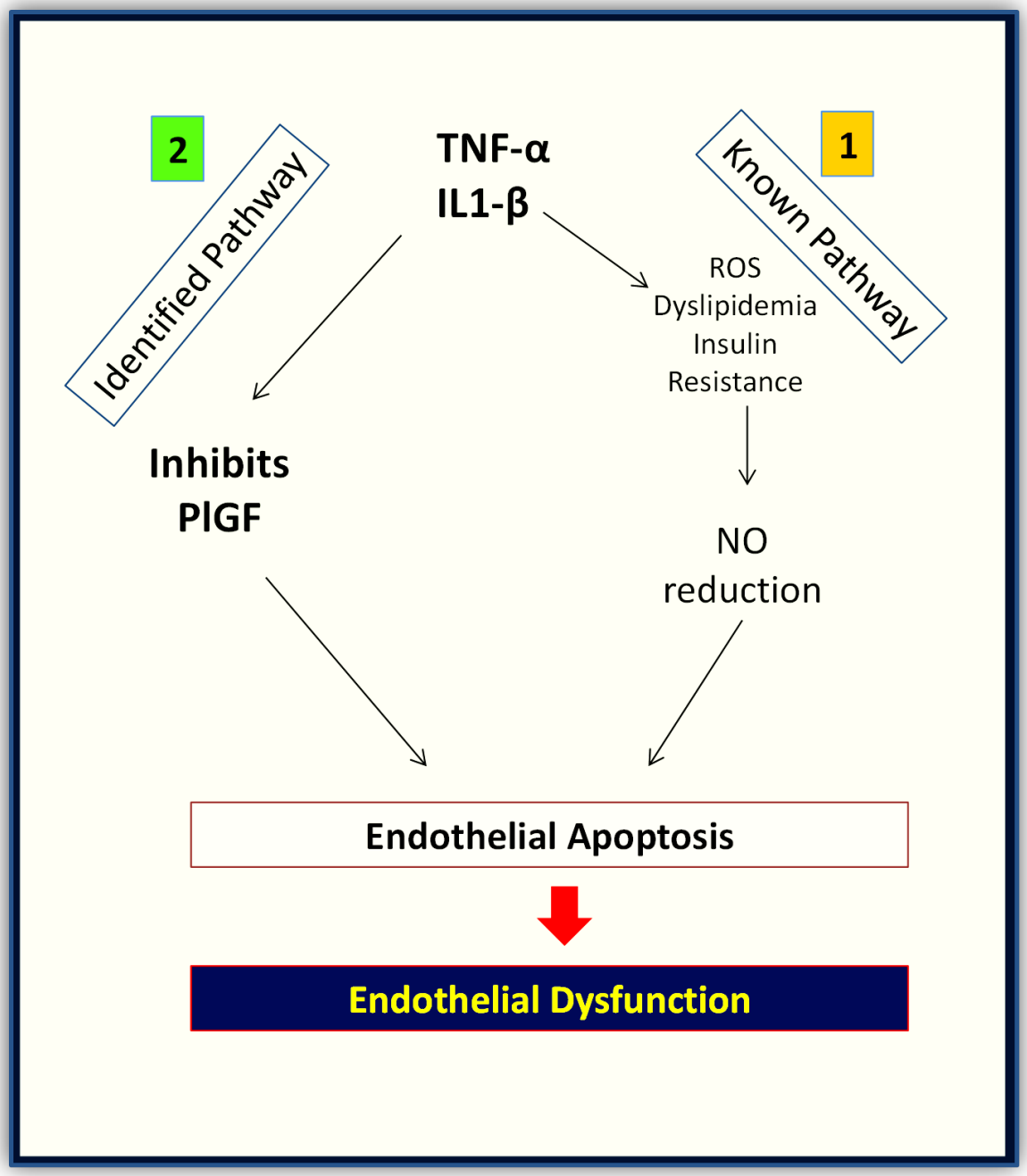


Figure 7.21 Loss of PlGF: An intermediary factor in the process of local Inflammation to systemic endothelial dysfunction. *(1). Known Pathway – TNF-α has a central role in EC apoptosis, linked to endothelial dysfunction resulting in SVD in several disorders [17, 22, 27]. Abnormal high levels of pro-inflammatory cytokines, TNF-α, IL1-β leads to several metabolic derangements, such as ROS, lipid abnormalities, decrease in bioavailability of NO, which sets the stage for EC apoptosis. EC apoptosis is reported to be one of the factors contributing to endothelial dysfunction [78, 79]. (2). Identified pathway - TNF-α, IL1-β inhibit PlGF via a transcriptional regulation mechanism. Loss of PlGF induced EC apoptosis (Present study).*

Chapter 8 General Discussion and future work

General Discussion

In this thesis I identify the functional link between the regulation of endothelial PlGF expression in response to potent angiogenic factor FGF-2, and the inflammatory cytokine TNF-α. The effects of these interplays between angiogenic factors and cytokines in PlGF-mediated angiogenesis and EC functions were examined in this study. Establishing the signalling pathways governing the regulatory mechanisms in PlGF secretion under the influence of FGF-2 and TNF-α could provide cues to compare the significant and temporal dynamic variances that might exist; for example, differential regulatory mechanisms in VEGF or FGF-2 induced PlGF in ECs and its effects on EC functions. My data would have major implications to further the understanding of the role of endothelial PlGF in dysregulation underlying inflammatory disorders of acute and chronic in nature, and subsequently the scope to improve therapeutic angiogenesis.

Effects of various stimuli on endothelial PlGF regulation and EC functions.

The molecular mechanisms and factors that lead to neovascularisation processes, angiogenesis and/or arteriogenesis, are diverse. However, blood vessel growth is accepted to be solely dependent on the function of ECs (Carmeliet 2000, M 2011). Being the interface between blood and tissue, ECs remain the main regulator of vascular homeostasis. Combined data from our observations in line with previous reports (Yonekura, Sakurai et al. 1999, Fischer, Jonckx et al. 2007, Dewerchin and Carmeliet 2012, Kim, Cho et al. 2012) demonstrate ECs to be an important source of PlGF. PlGF is redundant in normal physiology. However, it is identified as a key mediator and disease modifying agent for its predominant role in the angiogenic and inflammatory switch in several pathologies (Carmeliet 2000, Chen, Hsieh et al. 2004, Cianfarani, Zambruno et al. 2006, Dewerchin and Carmeliet 2012). A series of

studies highlighted the role of PlGF in multiple pathologies, including tissue ischemia, cancer and inflammation (Dewerchin and Carmeliet 2012). PlGF had beneficial or harmful effects basing on the stage of disease progression.

FGF-2-treated ECs amplified PlGF expression at both protein and mRNA levels (Current study), unlike PlGF production in response to VEGF that followed a post-transcriptional mechanism (Yao, Yang et al. 2005). It is also noteworthy that VEGF (25 ng/ml) amplified PlGF protein levels (Yao, Yang et al. 2005) at a higher concentration than FGF-2 (10 ng/ml)-mediated PlGF expression in HUVECs. Also, the concentration of VEGF (100 ng/ml) used to characterise the signalling pathways involved in VEGF-mediated PlGF secretion (Yao, Yang et al. 2005) being ten times greater than FGF-2 (10 ng/ml). Differential effects of FGF-2 and VEGF on PlGF secretion in ECs existed (Table 8.1)

Unlike VEGF, PlGF (Carmeliet, Moons et al. 2001, Luttun, Tjwa et al. 2002, Iwama, Uemura et al. 2006, Kolakowski, Berry et al. 2006) and FGF-2 (Unger, Banai et al. 1994, Laham, Chronos et al. 2000, Lazarous, Unger et al. 2000, Coenegrachts, Maes et al. 2010, Wu, Wu et al. 2010, Schmidt, Kharabi Masouleh et al. 2011) reported to be both pro-angiogenic and pro-arteriogenic without any deleterious side effects, such as tumour growth or heart, limb and ocular ischemia (Carmeliet and Conway 2001, Luttun, Brusselmans et al. 2002, Pipp, Heil et al. 2003, Rakic, Lambert et al. 2003, Dewerchin and Carmeliet 2012). Both FGF-2 and PlGF have been used as therapeutic agents in clinical trials to stimulate revascularization for the treatment of chronic ischemic conditions (Losordo, Vale et al. 1998, Laham, Sellke et al. 1999, Ruel, Laham et al. 2002, Simons, Annex et al. 2002) (Tables 1.2 -1.3).

FGF-2-induced PlGF (Current study)	VEGF-2-induced PlGF (Yao. 2005)
❑ Transcriptionally regulated with mRNA amplification	❑ No changes in mRNA levels, post-transcriptional modification
❑ FGF-2 concentration used - 10 ng/ml	❑ VEGF concentration used - 100 ng/ml
❑ ERK1/2 is completed abrogated FGF-2-induced PlGF to the basal levels	❑ Partial suppression of VEGF-induced PlGF levels in the presence of ERK1/2 inhibitor
❑ PKC isoforms involved - PKC α, δ, ε	❑ PKC isoforms involved - PKC-β
❑ Src, Akt required for basal and FGF-2 mediated PlGF secretion	❑ Src, Akt had no significant effect

Table 8.1 Differential regulation of FGF-2 and VEGF-induced PlGF levels in ECs

In contrast, when VEGF was administered as a therapeutic angiogenic factor it was reported to have adverse effects, such as Endothelium-derived relaxing factor (EDRF)-mediated hypotension (Hariawala, Horowitz et al. 1996), tachycardia, decreases in cardiac output and stroke volume, changes in preload and after-load (Yang, Bunting et al. 2000), a high rate of failure to thrive/death and formation of endothelial cell-derived intramural vascular tumours in the implantation site (Lee, Springer et al. 2000). These results undermine VEGF as a therapeutic agent; in contrast, FGF-2 has been suggested as a safer option with therapeutic potential for neovascularisation trials (Laham, Sellke et al. 1999, Laham, Chronos et al. 2000, Lederman, Mendelsohn et al. 2002, Simons, Annex et al. 2002). We believe that findings of these differential effects in the regulatory mechanisms between VEGF and FGF-2 on PlGF levels would contribute to a better understanding of the roles of the growth factors, to effectively harness their potential as target therapeutic molecules.

Research interest has been focused on the potential administration of PlGF and FGF-2 to alleviate conditions characterised by insufficient blood supply, such as MI, limb ischaemia

and PVD (Table 1.3). The rationale of this study was to focus on the interaction between PlGF and FGF-2, centering on their overlapping beneficial functions and their potential as target molecules for neovascularisation therapies (Tables 1.2. 1.3) (Figure 6.1). We demonstrate that FGF-2 amplifies PlGF mRNA, yet PlGF up regulation by VEGF is reported to be a post-transcriptional modification (Yao, Yang et al. 2005). Up-regulation of PlGF mRNA and protein was observed in pathological angiogenesis in several disorders, including MI [5], diabetic retinopathy [41], brain tumours [42] and retinal ischaemic disorders [43].

FGF-2-induced PlGF is mediated via MAPK and multiple isoforms of PKC. VEGF-induced PlGF involved PKC-β and partial activation of MAPK (Yao, Yang et al. 2005). Similarly, in animal models, PKC and MAPK signalling cascades have been reported to be vital molecules in FGF-2-mediated prevention of ischemia/reperfusion injury (Padua, Sethi et al. 1995, Padua, Merle et al. 1998, House, Melhorn et al. 2007). A detailed involvement of PKC isoforms, especially PKC-α, β, δ and ε in cellular and pathophysiological conditions has been discussed in Chapter 5. We also demonstrated FGF-2 stimulates PLD signalling via PKC activation in ECs, independent of PLCγ (Ahmed, Plevin et al. 1994). Elevated PLD activity has been reported in several cancer tissues and is implied in tumour cell proliferation and survival (Pedrono, Saiag et al. 2007). As PLD is involved in FGF-mediated PlGF, and also cancer progression where FGF-2 as well as PlGF contribute to disease progression, the effect of inhibiting PLD as a common targeting molecule can be examined *in vitro*; this is an option to explore with future application of this current research. FGF-2 up-regulates VEGF expression in ECs through autocrine and paracrine regulation in endothelial and non-ECs (Seghezzi, Patel et al. 1998). However, recent work from our laboratory demonstrated VEGF to amplify sVEGFR-1 expression by the activation of VEGFR-1 gene promoter (Ahmad, Hewett et al. 2011). This link between VEGF and sVEGFR-1 (Ahmad, Hewett et al. 2011)

raises the question of the role of VEGF in FGF-2 amplified PlGF and the EC functions in the current study, as FGF-2 is reported to amplify VEGF expression in ECs (Seghezzi, Patel et al. 1998). VEGF detection in a range of untreated ECs was undetectable (Figure 4.2).

NO produced from eNOS plays an important role during physiological and pathological EC functions via regulation of anti-apoptosis, pro-angiogenesis and other mechanisms. *eNOS* $^{-/-}$ in mice impairs angiogenesis function *in vivo* [19,20]. FGF2, but not VEGF, stimulated eNOS expression in a time- and concentration-dependent manner in four different ovine foeto-placental artery endothelial (oFPAE) cells (Zheng, Bird et al. 1999). FGF2 and VEGF differ greatly in the activation pattern of ERK (Mata-Greenwood, Liao et al. 2008). In oFPAE cells, FGF2 stimulated eNOS expression at all doses (1, 10, 100 ng/ml) tested, and this stimulation maximised with 10 ng/ml FGF-2 (Mata-Greenwood, Liao et al. 2008). Time course experiments in the same study demonstrated a significant increase in eNOS protein at 12 h and 24 h, with a maximal effect at 24 h (Mata-Greenwood, Liao et al. 2008). FGF2 provoked a sustained phosphorylation (5 min to 12 h), unlike VEGF which only stimulated transient (5 min) ERK phosphorylation (Mata-Greenwood, Liao et al. 2008). This data of eNOS up-regulation is in accordance with the kinetics (concentration and time) of FGF-2-induced PlGF in the current study. This clearly explains the effects of FGF-2-induced *in vitro* tube formation in ECs. However, in order to demonstrate endogenous PlGF regulation of eNOS, in our experimental model, HUVEC with inactivated PlGF or protein/tissue from *plgf* $^{-/-}$ mice, are to be subjected to detection of NO-eNOS activity by western blotting/ELISA for protein analysis or Northern blotting/QPCR to observe changes in mRNA levels. Although ERK is reported to mediate the release of NO/eNOS in ECs in response to PlGF (Shen and Chen 2008), FGF-2 (Mata-Greenwood, Liao et al. 2008) and VEGF (Mata-Greenwood, Liao et al. 2008), differential regulation of ERK molecule, at least in FGF-2 and VEGF, was noted. Wherein,

FGF-2 induced a stronger activation of ERK and sustained activation (up to 12 h), VEGF only induced a transient and weak activation (5-10 min) (Mata-Greenwood, Liao et al. 2008). In 2006, we had demonstrated that VEGFR-1 is the signalling receptor for eNOS activation and *in vitro* tube formation (Ahmad, Hewett et al. 2006). Furthermore, Akt appeared to be the common mediator in VEGF-stimulated eNOS activation and *in vitro* angiogenesis for VEGF receptor (VEGFR-1 and VEGFR-2) pathways (Ahmad, Hewett et al. 2006). In the light of differential activation of multiple signalling pathways dictating FGF-2 and VEGF mediated eNOS phosphorylation (Yao, Yang et al. 2005), to learn if such differential mechanisms govern FGF-2 and PlGF induced NO-eNOS awaits confirmation in our experimental setting. This could be achieved by treating cultured HUVECs with FGF-2 (1 to 100 ng/ml) for a range of time points (up to 24 hours) or pre-treated with signalling inhibitors involved in FGF-2-induced PlGF expression, such as ERK, PKC, PLD and PI3K. Assessing for NO concentrations in these experimental conditions using Western blotting or QuantiChrom NO Assay Kit, would aid in understanding the details of the differential effects of these growth factors and their effects in vascular insufficient conditions, such as foetal growth restriction, PE or CVD. We have shown the requirement of PlGF in FGF-2-mediated angiogenesis by *in vitro* tube formation and aortic ring assay models. FGF-2-mediated angiogenesis was truncated in aortic ring assay performed with tissue from *plgf*$^{-/-}$ mice when compared to the wild type. The significant findings of this study lead to the proposition of the FGF-2/PlGF autocrine and paracrine loops that may regulate the angiogenic switch, and could contribute to ameliorate vascular complications in diverse angiogenic conditions including MI. FGF-2 also has been shown to induce the up regulation of endothelial VEGF in an autocrine manner (Seghezzi, Patel et al. 1998). Besides, systemic overexpression of VEGF-A in mice and HUVEC resulted in significantly elevated circulating sVEGFR-1 expression (Ahmad, Hewett

et al. 2011). These findings raise the question whether the increase in PlGF levels is a direct effect of FGF-2, or a secondary effect of FGF-2-induced VEGF on PlGF, or both. If so, the inhibition of FGF-2 mediated angiogenesis in the absence of PlGF is the secondary effect of VEGF-induced sVEGFR-1, which demands further investigation in our experimental settings. It is beyond the scope of this study to extrapolate the explanation in the light of the involvement of VEGF. More signalling and EC functional studies are needed to dissect the mechanisms for selectivity amplification of PlGF, by FGF-2 or VEGF, which are currently vague, a hypothesis that awaits further confirmation in our cell system.

Signalling molecules mediating FGF-2-induced PlGF in ECs, PKC, ERK1/2 and PI-3K pathways contributed to PlGF up-regulation in ECs and human umbilical artery SMCs (HUASMCs) (Pan, Fu et al. 2010) in response to Ang II, establishing a role of PlGF in mediating Ang II-induced proliferation in vascular ECs and SMCs (Pan, Fu et al. 2010). Angiotensin II (Ang II), a significant contributor to the pathobiology of atherosclerosis and vascular disease, is a bi-functional growth factor stimulating proliferative and anti-proliferative pathways simultaneously (Dimmeler, Rippmann et al. 1997). In ECs, Ang II mediates inhibition of FGF-2-induced EC proliferation via stimulation of its receptor AT_2 (Stoll, Steckelings et al. 1995). This suggests that using these inhibitors to manipulate PlGF will, therefore, bring about changes in Ang II-mediated ECs and SMCs functions as well.

Although common signal transducing molecules mediating EC functions have been identified, the major challenge is to segregate one mechanism from another, as these growth factors and the pathways governing growth factor regulation could manifest autocrine, paracrine or intracrine effects on cellular responses. This could result in unwanted cellular responses, such as endothelial dysregulation or dysfunction leading to chronic inflammation.

In order to effectively harness the potential of these growth factors, an improved understanding and identifying the crucial genes activated by the PlGF, FGF-2 and VEGF that elicit the signal transduction mediating the complex biological functions of VEGF and FGF-2 amplified PlGF will be essential.

Chronic inflammation is a hallmark of and a key factor in the pathogenesis of endothelial dysfunction (Murdaca, Colombo et al. 2012). Anti-cytokine therapy influences angiogenesis. I demonstrated with substantial evidence that inflammatory cytokines TNF-α, IL-1β and INFγ have an inhibiting effect on PlGF protein levels (Current study). TNF-α and IL-1β are extremely potent inflammatory molecules, besides being the primary mediators of septic shock (Feghali and Wright 1997). I showed that TNF-α suppression of PlGF is not only time- and concentration-dependent but also regulated at a transcriptional level. Vascular endothelium is known to be a specific target of TNF-α (Chia, Qadan et al. 2003, McKellar, McCarey et al. 2009, Zhang, Park et al. 2009) causing widespread vascular damage/disruption to the endothelium (Mutunga, Fulton et al. 2001). TNFR1 and TNFR2 activate inflammation as well as VEGFR2, promoting angiogenesis; a hallmark of chronic inflammation (Review) (Pober and Sessa 2007). Moreover, inflammatory response of PlGF involves recruitment of inflammatory cells, pericytes and SMCs (Adini, Kornaga et al. 2002, Fischer, Jonckx et al. 2007, Dewerchin and Carmeliet 2012). In contrast to ECs, it was interesting to note that SMCs had elevated PlGF levels in response to TNF-α. It is tempting to speculate that the differential effect of TNF-α on PlGF levels in ECs and SMCs may contribute towards the imbalance of EC/SMC activators or inhibitors that have been suggested to be associated with occlusive disorders such as PE (Dubey, Jackson et al. 1997, Dubeyl, Rosselli et al. 2000). However, this hypothesis awaits further confirmation in our cell system.

Our group previously reported that PlGF up regulates the anti-apoptotic enzyme bcl-2 and sustained tube formation in HUVEC (Cai, Ahmad et al. 2003). In the current study, TNF-α inhibited angiogenesis was restored by addition of exogenous FGF-2 and PlGF, in line with our findings previously (Cai, Ahmad et al. 2003). Infliximab, a chimeric monoclonal antibody, and approved treatment for inflammatory disorders, including, RA and Crohn's disease specifically neutralises the anti-inflammatory effect of TNF-α (Maini and Feldmann 2002). Of special relevance to angiogenesis, infliximab reduced VEGF expression within the arthritic synovium and a reduction in the number of blood vessels in infliximab-treated patients. Hence anti-TNF-α therapy does not restore a long-lasting remission; instead its perpetuation continues the cytokine imbalance, leading to relapse of disease upon withdrawal of the therapy (Review) (Maini and Feldmann 2002).

Women with PE have increased TNF-α level and reduced PlGF. These women and their children are reported to be at a higher risk of developing CVD at a later stage in life (Bellamy, Casas et al. 2007, Thomas 2014). TNF-α, EC apoptosis and endothelial dysfunction are common denominators of several acute and chronic inflammatory disorders, associated clinically with excessive cardiovascular risk and higher mortality than the general population (Bellamy, Casas et al. 2007) (Table 6.1). In the current studies, TUNEL assay analyses showed that loss of PlGF induced EC apoptosis that could not be reversed by growth factors. We believe that the reduced formation of the capillary net in PlGF gene-inactivated cells is perhaps a secondary effect to EC apoptosis.

The endothelium is the largest secretory organ in the body, with a surface area comparable to approximately six tennis courts. (Cooke 2000). Hence any subtle changes mark a noticeable impact. ECs are proposed to be an important source of PlGF, manifesting cellular activities in autocrine and paracrine regulation. Multiple approaches can achieve modulation of neovascularisation processes; either by blocking the action of pro-angiogenic intermediaries, such as the signalling molecules involved in FGF-2-mediated PlGF, or to improve anti-pro-inflammatory cytokine therapies that may inhibit PlGF, or vice versa. In addition, future benefits may be gleaned by working with the common signalling molecules shared between neovascularisation and inflammation to inhibit or enhance their response in inflammatory angiogenesis. My study provides developments to understand PlGF regulation and subsequent involvement in the processes of inflammatory angiogenesis, either by delivering or blocking endothelial PlGF, via FGF-2 or inflammatory cytokines such as TNF-α, IL-1β or INFγ respectively. Pro-inflammatory mediators, such as TNF-α, suppress endothelial PlGF, and loss of PlGF leads to EC apoptosis (current study). Future work to examine whether TNF-α-suppressed PlGF leads to EC apoptosis in a disease model is warranted. Moreover, EC apoptosis is known to contribute to endothelial dysfunction, assessing whether the loss of PlGF-induced EC apoptosis leads to endothelial dysregulation remains to be proved in our experimental model. Additionally, the signalling molecules mediating EC apoptosis in the presence or absence of inflammatory mediators needs to be designed, which would aid in understanding the mechanisms and the role of PlGF-manifested EC apoptosis and/or endothelial dysfunction in acute or chronic inflammatory disorders if exists.

In addition to being identified as a pro-angiogenic and pro-arteriogenic factor (Dewerchin and Carmeliet 2012), PlGF is known to promote atherogenic neointima formation, macrophage

accumulation, neovascularisation and EC activation (Khurana, Moons et al. 2005, Dewerchin and Carmeliet 2012). Short-term delivery of anti-PlGF antibody treatment to ApoE-deficient (apoE(-/-)) mice, appeared to act more efficiently during the early phase of atherogenesis, by reducing early atherosclerotic plaque size and inflammatory cell infiltration in the lesion (Roncal, Buysschaert et al. 2010). By contrast, in the same study CD4:TGFbetaRII (DN) x apoE(-/-) mice, a more severe atherosclerosis model, the anti-PlGF antibody was ineffective (Roncal, Buysschaert et al. 2010).

Patients with ACS, including AMI, have high plasma PlGF level within 4-24 hours of symptom onset has positively correlated with >60% improvement in LVEF (Iwama, Uemura et al. 2006), in line with several studies that demonstrated the cardio-protective function of PlGF (Table 1.2). PlGF involvement in pathophysiology during the late symptomatic phase of ACS could differ from that during early disease (Kim, Cho et al. 2012). PlGF-mediated angiogenesis is amplified in response to hypoxic conditions involving cardiomyocytes and fibroblasts, which act in a paracrine manner on ECs contributing to myocardial angiogenesis (Torry, Tomanek et al. 2009). This process also involves PlGF promoted chemotaxis and recruitment of macrophages and monocytes enhancing tissue healing (Torry, Tomanek et al. 2009). Consequently, PlGF is bi-functional; it could be beneficial or deleterious depends on the stage of disease progression. Based on the above-stated findings and the contributing factors of SMC and leukocyte recruitment in atherosclerosis, there is a suggestion that the differential effects of inflammatory cytokines that are up regulated to abnormal levels (Table 1.6) in a disease state may aid to learn the hidden consequences during chronic inflammatory disorders, including atherosclerosis. Though the data is very preliminary in nature, the differential effects of TNF-α on EC and SMC secreted PlGF that is observed in this study,

wherein endothelial PlGF was suppressed and SMC secreted PlGF was amplified (Current study), is a hypothesis that awaits further confirmation in our experimental model. Based on the evidence of the *in vitro* findings the study could be advanced to *in vivo* analysis.

PlGF promotes VEGF-mediated angiogenesis in a number of ways. PlGF is thought to bind to VEGFR-1, and trigger the downstream signalling to enhance VEGF signalling via VEGFR-2 (Luttun, Autiero et al. 2004). PlGF displaces VEGF from VEGFR-1, thus making VEGF available for VEGFR-2 interactions (Carmeliet, Moons et al. 2001) or by forming VEGF-PlGF heterodimers (Cao, Chen et al. 1996). The role of VEGF in PlGF-mediated cellular functions is vital. VEGF also enhances the expression of sVEGFR-1 *in vitro* as well as *in vivo* via autocrine regulation (Ahmad, Hewett et al. 2011). It is very puzzling and beyond the scope of the current study to extrapolate the exact role of VEGF and needs to examine VEGF, VEGFR-1 and sVEGFR-1 interactions in our experimental model.

A number of soluble and cell-bound factors may stimulate or inhibit neovascularisation. Angiogenesis is a finely tuned note balancing between pro-angiogenic and anti-angiogenic mediators, like growth factors, cytokines, chemokines, cellular adhesion molecules and others (Cooke 2000, Szekanecz, Besenyei et al. 2010, Kim, Cho et al. 2012). Formation of vascular networks is a complex process and involves coordinated workings of multiple growth factors and their signalling peptides, which need a thorough understanding of their role in autocrine and paracrine regulation. With PlGF being non-redundant in pathological angiogenesis, henceforth it needs timely controlled regulation of PlGF to be taken into serious consideration to modulate PlGF and/or its receptor, VEGFR-1, as potential therapeutic tools. Our studies delineating the distinct signalling enzymes and their pathways involved in FGF-2-induced PlGF release and inflammatory mediator effect on suppressed endothelial PlGF that may well identify new therapeutic targets for the treatment of various pathologies.

Chapter 9 Final Conclusion

The current study and previous lines of evidence demonstrate PlGF to be the target for multiple molecules, including growth factors such as VEGF (Yao, Yang et al. 2005) and its isoforms (Current study Chapter 4), Ang II (Pan, Fu et al. 2010), and FGF-2 (Current study chapters 5 and 6). These growth amplify PlGF expression. Conversly, PlGF is also the target for inflammatory mediators like TNF-α, IL-1β or INFγ which suppress PlGF expression (Current study chapter 7). Therefore, manipulation of PlGF and its VEGFR-1 that mediate pathological angiogenesis is not only an ideal target, but a very decisive target that warrants careful examination in the regulation of its expression in different types of endothelial and non-ECs origin, on which PlGF and VEGFR-1 manifest their effect in multiple ways. The findings of this study, therefore, aid in understanding the modulation of PlGF in response to various stimuli, the potential benefit to target therapies and the possibility to alleviate disease.

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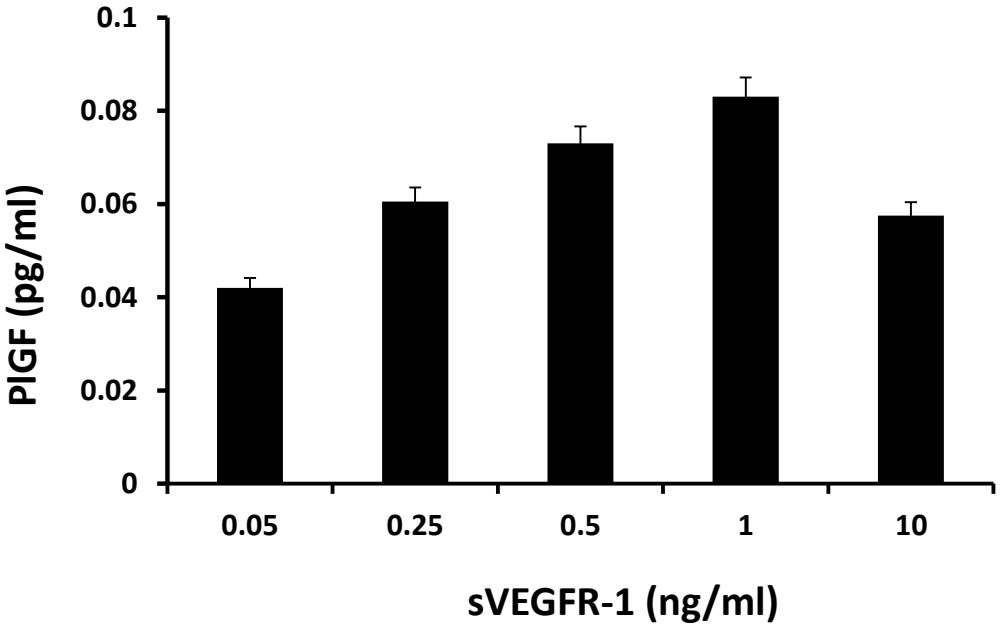
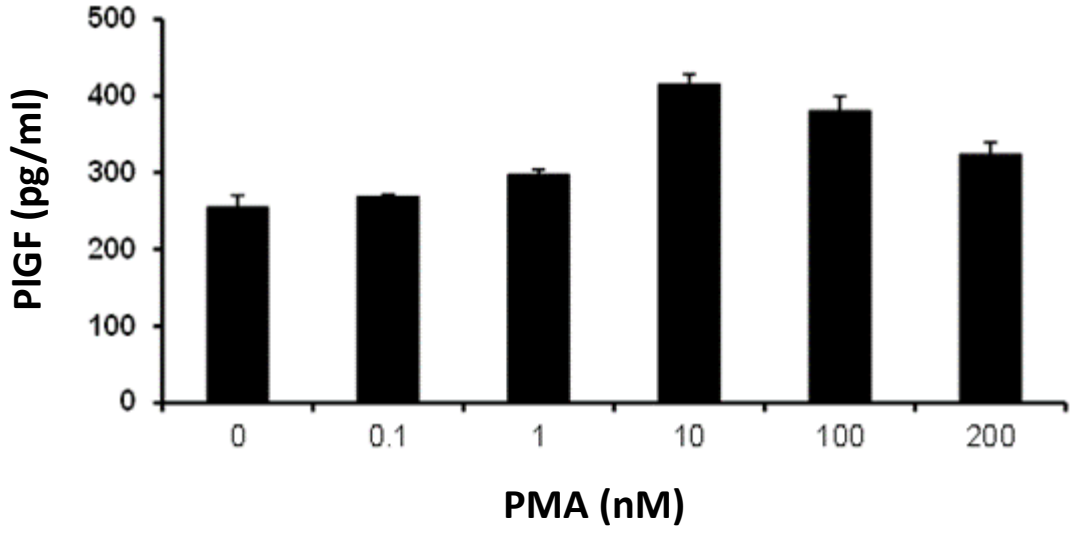
Appendices

Appendix I



Appendix II

PlGF levels in response to treatment with increasing concentrations of PMA in HUVEC



Appendix III

Chemical Reagents and Suppliers

1-Butanol:	Sigma, Poole, UK
2-Butanol:	Sigma, Poole, UK
5 x transcription buffer:	Promega, Southampton, UK
6-keto-PGF1α ELISA kit:	R & D Systems, Abingdon, UK
α-APP:	Calbiochem, Nottingham, UK
AAV helper free system:	Stratagene, UK
Acetic acid [glacial]:	Sigma, Poole, UK
Acrylamide solution [40 %]:	Bio-Rad, UK
Agar select:	Sigma, Poole, UK
Agarose:	Sigma, Poole, UK
Ammonium persulphate:	Sigma, Poole, UK
Angiokit:	TCS Biologicals, Buckingham, UK
Angiopoietin1:	R & D Systems, Abingdon, UK
Angiopoietin2:	R & D Systems, Abingdon, UK
BCA protein assay:	Bio-Rad, UK
Bovine serum albumin:	Sigma, Poole, UK
Bromophenol blue:	Sigma, Poole, UK
Calcium chloride:	Sigma, Poole, UK
Carbon dioxide:	Air products, Walton on Thames, UK
Chloroform:	Sigma, Poole, UK
Collagen:	Sigma, Poole, UK

Colloidal Coomassie blue staining kit:	Novex, San Diego, CA
Competent DH5α cells:	Gibco, Paisley, Scotland
Cycloheximide:	Sigma, Poole, UK
Denhardt's reagent:	Sigma, Poole, UK
Dextran sulphate:	Sigma, Poole, UK
Diethyl Pyrocarbonate [DEPC]:	Sigma, Poole, UK
Diff-Quik staining solution:	Dade Behring Ltd, UK
DMEM [phenol red free]:	ICN, Basingstoke, UK
DMSO:	Sigma, Poole, UK
DTT:	Pharmacia, Herts, UK
ECL detection kit:	Amersham, Buckinghamshire, UK
EDTA:	Sigma, Poole, UK
EGF:	ReliaTECH, Braunschweig, Germany
Ethanol [99.7-100 %]:	BDH, Poole, UK
Ethidium bromide:	Sigma, Poole, UK
FGF:	ReliaTECH, Braunschweig, Germany
Foetal calf serum:	Gibco, Paisley, Scotland
FURA2:	Sigma, Poole, UK
Gelatin:	Sigma, Poole, UK
GF109203X:	Calbiochem, Nottingham, UK
Glycerol:	Sigma, Poole, UK
Glycine:	Sigma, Poole, UK
Goat serum:	Sigma, Poole, UK

Gö6976:	Calbiochem, Nottingham, UK
GW5074:	Calbiochem, Nottingham, UK
Ham F12:	ICN, Basingstoke, UK
Heparin:	Sigma, Poole, UK
HEPES:	Sigma, Poole, UK
Hispidin:	Calbiochem, Nottingham, UK
Hybond N+ nylon membrane:	Amersham, Buckinghamshire, UK
Hydrogen chloride:	Sigma, Poole, UK
Hydrogen peroxide:	Sigma, Poole, UK
Imidazole:	Sigma, Poole, UK
Isohelenin:	Calbiochem, Nottingham, UK
Isopropanol:	Sigma, Poole, UK
Kaleidoscope prestained standards:	Bio-Rad, UK
Kodak, X-Omat AR film:	Sigma, Poole, UK
KT-5823:	Calbiochem, Nottingham, UK
L-Glutamine:	Sigma, Poole, UK
Leupeptin:	Sigma, Poole, UK
L-NNA [N^G-NO_2-L-Arginine]:	Calbiochem, Nottingham, UK
LY294002:	Calbiochem, Nottingham, UK
Magnesium sulphate:	Sigma, Poole, UK
Marvell dried milk:	Sainsbury's, Birmingham, UK
Matrigel [growth factor-reduced]:	BD Biosciences, Cowley, UK
Mayer's Haematoxylin:	Sigma, Poole, UK

Hispidin:	Calbiochem, Nottingham, UK
M199 medium:	ICN, Basingstoke, UK
Methanol:	Sigma, Poole, UK
Microcarrier beads:	Sigma, Poole, UK
Mineral oil:	Sigma, Poole, UK
MG262:	Calbiochem, Nottingham, UK
MTT:	Sigma, Poole, UK
Murine sVEGFR-1 ELISA Duoset:	R & D Systems, Abingdon, UK
NP40:	Sigma, Poole, UK
ODQ:	Calbiochem, Nottingham, UK
Owrens buffer:	Sigma, Poole, UK
PCR loading dye:	Sigma, Poole, UK
Penicillin:	Gibco, Paisley, Scotland
Pepstatin A:	Sigma, Poole, UK
Phenol:chroroform:	Sigma, Poole, UK
Phenylmethylsulfonyl fluoride [PMSF]:	Sigma, Poole, UK
Phosphate buffered saline tablets [PBS]:	Sigma, Poole, UK
Phosphatidic acid:	Calbiochem, Nottingham, UK
PlGF-1:	ReliaTECH, Braunschweig, Germany
PMA:	Calbiochem, Nottingham, UK
Potassium chloride:	Sigma, Poole, UK
PP2:	Calbiochem, Nottingham, UK
Proteinase K:	Boehringer Mannheim, Sussex, UK

Protein A-agarose:	Sigma, Poole, UK
Protein G-agarose:	Sigma, Poole, UK
QIAprep-spin Plasmid Kit:	Qiagen, Surrey, UK
QIAquick extraction kit:	Qiagen, Surrey, UK
Rapamycin:	Calbiochem, Nottingham, UK
Restriction enzymes:	Amersham, Buckinghamshire, UK
RNase A:	Sigma, Poole, UK
Ro 320432:	Calbiochem, Nottingham, UK
RPMI 1640:	Gibco, Paisley, Scotland
Shrimp Alkaline phosphatase:	NEB, UK
Silica gel:	Sigma, Poole, UK
Sodium acetate:	Sigma, Poole, UK
Sodium chloride:	Sigma, Poole, UK
Sodium citrate:	Sigma, Poole, UK
Sodium dodecyl sulphate:	Sigma, Poole, UK
Soluble VEGFR-1 receptor-Fc:	R & D Systems, Abingdon, UK
Sodium hydroxide:	Sigma, Poole, UK
Streptomycin:	Gibco, Paisley, Scotland
T4- DNA ligase:	NEB, UK
TC-100 medium:	Gibco, Paisley, Scotland
TEMED:	Bio-Rad
Thymeleatoxin:	Calbiochem, Nottingham, UK
Triethanolamine:	Sigma, Poole, UK

Tris:	Sigma, Poole, UK
Triton X-100:	Sigma, Poole, UK
Trypsin:	Sigma, Poole, UK
Tween-20:	Sigma, Poole, UK
U0126:	Calbiochem, Nottingham, UK
U73122:	Calbiochem, Nottingham, UK
VEGF-A:	ReliaTECH, Braunschweig, Germany
VEGF-E	ReliaTECH, Braunschweig, Germany
VEGF-A:PlGF-1:	ReliaTECH, Braunschweig, Germany
Xylene:	Surgipath, Cambridgeshire, UK

All other cell culture reagents and chemicals were obtained from Sigma, Poole, UK.